From Department of Cell and Molecular Biology
Karolinska Institutet, Stockholm, Sweden

NOTCH SIGNALING REQUIEM: ORCHESTRAL ROLE OF NOTCH SIGNALING IN CANCER AND DEVELOPMENTAL DISEASE

TSOI, Yat Long
蔡日朗

Karolinska Institutet

Stockholm 2020

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Published by Karolinska Institutet.
Printed by US-AB.

ISBN: 978-91-7831-916-9

Notch Signaling Requiem: Orchestral Role of Notch Signaling in Cancer and Developmental Disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

TSOI Yat Long

Principal Supervisor:
Professor Urban Lendahl
Karolinska Institutet
Department of Cell and Molecular Biology

Co-supervisor(s):
Professor Kenneth R. Chien
Karolinska Institutet
Department of Cell and Molecular Biology

Opponent:
Professor Anna Bigas
Institut Hospital del Mar d'Investigacions Mèdique,
Barcelona, Spain

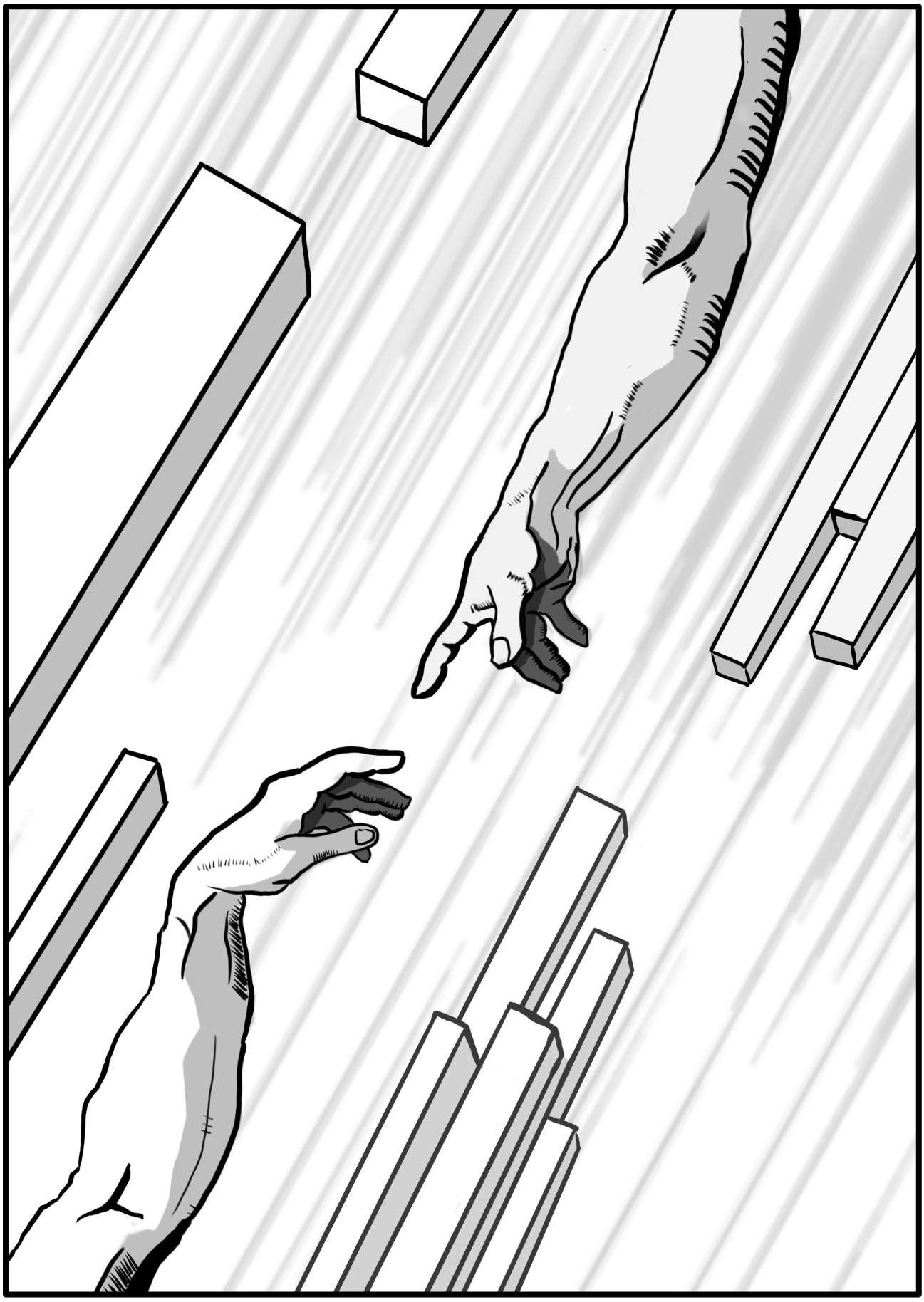
Examination Board:
Professor Marie Arsenian-Henriksson
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Professor Jonas Muhr
Karolinska Institutet
Department of Cell and Molecular Biology

Professor Mattias Mannervik
Stockholm University
Department of Molecular Biosciences

When I consider thy heavens,
The work of thy fingers,
The moon and the stars,
Which thou hast ordained;
What is man, that thou art mindful of him?
And the son of man, that thou visitest him?
- Pslam 8

Dedicated to my Homeland Hong Kong
A Requiem for our Sacrificed Souls



"*The Contact*", Sunny Tsoi, 2020. Inspired by "*Creation of Adam*" by Michelangelo and the interacting Notch receptors and ligands.

Abstract

Notch signaling is an evolutionary conserved contact-dependent cell-cell communication pathway. This "contact" spans from hydra to fruit flies to human; orchestrating development, homeostasis and cancer, thus the **Requiem**, a song of life and death. Upon the "contact" of Notch receptor and ligand, the intracellular domain NICD is released and translocates to the nucleus. NICD, together with the DNA binding protein CSL and other co-activators, activate downstream targets. In this thesis, I have investigated the role of Notch signaling in multiple contexts with a modular approach. This includes: the non-canonical role of CSL in breast cancer, crosstalk of Notch signaling with hypoxia signaling in cancer, canonical Notch signaling in blood development, a novel mouse model for Alagille syndrome, and the hyperactivated Notch during mammary development and tumourigenesis. Here I phrase them in five sections of a requiem (Mozart's Requiem, 1791):

Introitus: In **Paper I**, we found that ablation of CSL unleashed a hypoxic response in normoxic conditions and enhanced tumour growth in breast cancer. A large part of the deregulated genes in the CSL null cell line is Notch independent. We demonstrated a non-canonical role of CSL and the possible implication of loss of CSL in breast cancer.

Kyrie: In **Paper II**, we established that Notch signaling can modulate hypoxia signaling in multiple cancer cell types. By siRNA knocked down of HIF2α, we found that Notch signaling requires HIF2α for regulating a subset of Notch targets in medulloblastoma cells. Differences in the effect of N1ICD and N2ICD were also shown in the medulloblastoma cells. Lastly, we presented evidence of Notch signaling contributing to the HIF1α-to-HIF2α switch.

Dies Irae: In **Paper III**, we revealed that canonical Notch signaling is dispensable in adult steady-state and stress myelo-erythropoiesis by ablating CSL in the myeloid lineage. Some of the Notch targets were derepressed in some of the progenitor stages, indicating CSL could act as a repressor in some contexts.

Rex tremendae: In **Paper IV**, we established and characterized a mouse model for Alagille syndrome in human, recapitulating defects in multiple organ-systems. We showed a mutation in Jag1 caused delay differentiation and structural abnormalities in the bile ducts. From transcriptomics of mice and patients samples, we also found some commonly affected genes across species. Lastly, we discovered that the mutated Jag1 failed to bind to Notch1 and reduced the extent of Notch2 and Notch3 activation.

Lacrymosa: In **Paper V**, we observed that hyperactive Notch in the luminal lineage during lactation cause defect in ductal development and led to mammary tumour development. Furthermore, we showed that this lineage can contribute to a large part of the mammary tumour.

List of publications

Paper I

Loss of CSL Unlocks a Hypoxic Response and Enhanced Tumor Growth Potential in Breast Cancer Cells.

Braune EB*, **Tsoi YL*,** Phoon YP*, Landor S, Silva Cascales H, Ramsköld D, Deng Q, Lindqvist A, Lian X, Sahlgren C, Jin SB, Lendahl U.

Stem Cell Reports 2016 6;5 643-651

(* Co-first author)

Paper II

Notch signaling promotes a HIF2α-driven hypoxic response in multiple tumor cell types.

Mutvei AP, Landor SK, Fox R, Braune EB, **Tsoi YL,** Phoon YP, Sahlgren C, Hartman J, Bergh J, Jin S, Lendahl U.

Oncogene 2018 37;46 6083-6095

Paper III

Canonical Notch signaling is dispensable for adult steady-state and stress myelo-erythropoiesis.

Duarte S, Woll PS, Buza-Vidas N, Chin DWL, Boukarabila H, Luís TC, Stenson L, Bouriez-Jones T, Ferry H, Mead AJ, Atkinson D, Jin S, Clark SA, Wu B, Repapi E, Gray N, Taylor S, Mutvei AP, **Tsoi YL,** Nerlov C, Lendahl U, Jacobsen SEW.

Blood 2018 131;15 1712-1719

Paper IV

Mouse Model of Alagille Syndrome and Mechanisms of Jagged1 Missense Mutations.

Andersson ER, Chivukula IV, Hankeova S, Sjöqvist M, **Tsoi YL,** Ramsköld D, Masek J, Elmansuri A, Hoogendoorn A, Vazquez E, Storvall H, Netušilová J, Huch M, Fischler B, Ellis E, Contreras A, Nemeth A, Chien KC, Clevers H, Sandberg R, Bryja V, Lendahl U.

Gastroenterology 2018 154;4 1080-1095

Paper V

Notch activation in the mouse mammary luminal lineage leads to ductal hyperplasia and altered partitioning of luminal cell subtypes.

Phoon YP, Chivukula IV, **Tsoi YL**, Kanatani S, Uhlén P, Kuiper R, Lendahl U.

Experimental Cell Research 2020 *395*(1), 112156.

https://doi.org/https://doi.org/10.1016/j.yexcr.2020.112156

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List of abbreviations

ADAM	A disintegrin and metalloproteinase
AGM	Aorta-gonadmesonephros regions
ALGS	Alagille syndrome
ANK	Ankyrin repeats domain
bHLH	Basic helix-loop-helix
BMP	Bone morphological protein
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CAM	Chorioallantoic membrane
ChIP	Chromatin immunoprecipitation
CRISPR	Clustered regularly interspaced short palindromic repeats
CSL	CBF1/Suppressor of Hairless/LAG-1
CTCF	CCCTC-binding factor
DAPT	5-difluorophenylacetyl-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester
DEG	Differentially expressed genes
DLL	Delta-like
DOS	Delta/OSM-11 (DOS)
DSL	Delta, Serrate, Lag-2
ECD	Extracellular domain
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
FIH	Factor inhibiting HIF1α
Floxed	Flanked by loxP sites
gRNA	Guide-RNA
GO	Gene ontology
GSI	Gamma-secretase inhibitor
HD	Heterodimerisation domain
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia inducible factor
HRE	Hypoxia response element
HSC	Haematopoietic stem cell
ICD	Intracellular domain
IDE	Integrated Development Environment
IKK	IκB kinase
IL	Interleukin
IP	immunoprecipitation
IRES	Internal ribosomal entry site
KO	(Genetic) Knockout
LNR	Lin-12-Notch repeats

MAML	Mastermind-like
MET	Mesenchymal-to-epithelial transition
Mk	Megakaryocyte
MMTV	Mouse mammary tumour virus
MNNL	Module at the N-terminus of Notch Ligands
NERT2	Notch intracellular domain fused with estrogen receptor T2 variant
NF-κB	Nuclear factor of κB
NHEJ	non-homologous end joining
NRARP	Notch-regulated ankyrin repeat protein
NRR	Negative regulated region
N(1-4)ECD	Notch (1-4) extracellular domain
N(1-4)ICD	Notch (1-4) intracellular domain
NLS	Nuclear localization signal
OFT	Outflow tract
O-glycans	O-linked oligosaccharides
PAM	Protospacer adjacent motif
PEST	Proline (P), glutamic acid (E), serine (S), and threonine (T) rich
PGCC	Polyploid giant cancer cell
PHT	Primitive heart tube
pIIa	precursor of the sensory organ external cells
pIIb	precursor of the sensory organ internal cells
PKC	protein kinase C
PNC	Proneural clusters
PR	Progesterone receptor
PRC2	Polycomb repressive complex 2
PTA	Persistent truncus arteriosus
RAM	RBPJ-associated module
RBC	Red blood cell
RT-PCR	Reverse transcription polymerase chain reaction
SCC	Squamous cell carcinoma
scRNA-seq	Single cell RNA-sequencing
SHF	Second heart field
sgRNA	Single-guide-RNA
SOP	Sensory organ precursor
T-ALL	T-cell acute lymphoblastic leukemia
TAN-1	Translocation-associated Notch homolog1
TAD	Transactivation domain
TGF-β	Transforming growth factor β
TNBC	Triple negative breast cancer
TOF	Tetralogy of Fallot
VSMC	vascular smooth muscle cells

Introduction

This thesis addresses the importance of one of the most important signaling pathway – Notch signaling. "Balance" and "contact" are essential to Notch signaling, just as to our very existence. Over the billions years of our entire history of time, between lightness and darkness across billions light-years of our universe, there is one pale blue dot [a]. Everything on this pale blue dot was once forgettable star dust. Yet, in our pale blue dot, the star dust thrives as stardust crusaders [b], surviving and evolving with the song of life. "Perfectly balanced, as all things should be." [c] 'Twas the perfect balance of environmental conditions that made us. 'Twas also how we stand against the ever changing environment, to maintain a balance by reacting, regulating and relating to others, that made us.

The Chinese word "Chung Yung" (中庸), in English the "doctrine of mean", (or the strikingly similar Swedish word "lagom"), briefly represents the wisdom of being "just right" - not too much; not too little. A deeper meaning of Chung Yung is to do the right thing as who you are and at the right time. In living organisms, one key to balance is "cell signaling"; the communication of cells among themselves and to its environment. If cell signaling is compromised, the balance will be tilted and diseases will incur. For instance, excessive proliferation signal at the wrong time could possibly lead to cancer. In fact, many oncogenes fall into the category of signaling-related proteins, such as growth factors, G-proteins and kinases. On the other hand, inadequate signaling could lead to the underdevelopment of important tissues and organs. For example, defective Notch signaling could lead to underdevelopment of multiple organ systems in Alagille syndrome (will be discussed in **Paper IV**). Moreover, some signals play an important role in maintaining cell identity and behavior. Lack of such signal could also cause cancer. Thus, many tumour suppressor genes are signal-related.

Life has evolved complex languages of communication. Among the signaling pathways in the mammalian system, there is Notch signaling. It is highly evolutionary conserved, and involves in cancers and developmental diseases in human. This thesis contributes to the understanding of the roles and nature of Notch signaling in cancer and developmental diseases.

Cell Signaling

"Division of labor", the specialization of different individuals, was one of the key factors that enable the advances of human civilization. Similarly, our body adopted the division

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[a] *Pale Blue Dot* is a photograph of the earth taken by the Voyager 1 space probe 6 billion km from earth. It inspired astronomer Carl Sagan's book with the same name.

[b] Stardust crusader is the title name of the Japanese manga "*JoJo's Bizarre Adventure Part-3*" (Hirohiko Araki, 1989).

[c] A signature movie line from from "*Avengers: Infinity War*" (2018) by the main antagonist Thanos, who wish to wipe out half of the lives in the universe to make balanced world.

of labor in different cells, tissue and organ systems. Yet, we are many but one. Cells have to communicate and coordinate to maintain an organism. As complex languages have developed for the communication among humans, we have also evolved various complex signaling pathways to serve different purposes in multiple ranges.

There are multiple modes of action in signaling pathways, namely (a) intracrine: the signal produced and stay within the same cell; (b) autocrine: the signal secreted but act on the original cells; (c) juxtacrine: the signal stays on the cell membrane and signal adjacent cells by cell-cell contact; (d) paracrine: the secreted signal act on neighbouring cells; (e) endocrine: the secreted signal is released to the transport system and signal remote cells. Despite the diverse signaling pathways, possessing a great variety of properties and modes of action, each signaling pathway could be grossly categorized into four components:
Signal – the external signal that triggers a signaling pathway. Typically, it is a protein, lipids, ions, or other small molecules. Collectively, they are called ligands.
Receptor – the component responsible for receiving the signals.
Signal transduction – a series of biochemical events that would relay or sometimes amplify the signals. Typical signal transduction involves small molecules known as second messengers, or a series of protein interaction termed signal cascade.
Effector – the final component contributes to the response of the signaling pathway. It could be a transcription factor, a membrane channel protein or many other proteins responsible for different function in the cells.

It is these differences in the components that determine the numerous properties and modes of action in various signaling pathways. Furthermore, each component on its own may possess non-canonical functions and properties, such as modification and cross-talk with other pathways, expanding our study to a vast uncharted realm. In this thesis, we will take a modular approach to study the roles of different components of Notch signaling.

History of Notch signaling pathway

The term "notch" stemmed from a *Drosophila* mutant strain characterized by notches on the wings, first described by Dexter in 1914 [1]. Because of its sexual linkage nature and easily observed phenotype, notch mutants were used by Sir Thomas Hunt Morgan in his heredity study, which led to the understanding of the role of chromosome in heredity, and eventually his Nobel prize in 1933 [2,3]. Poulson was the first to study the phenotype in notch null embryos, carefully described the cytological differences, such as cell fate lineage switch in the ectoderm, opening a new door to study notch in developmental biology [4]. With the advances of molecular biology techniques in the 1980s, the *Drosophila* Notch gene was cloned and sequenced by Spyros Artavanis-Tsakonas' and Michael Young's [d] group independently [5,6]. The trans-membrane domain and the EGF-

[d] Michael Young was awarded the Nobel Prize in Physiology or Medicine in 2017 for his contribution to the understanding of the circadian rhythm.

repeats in the extracellular domain suggested that it is a membrane receptor. Not long after, Serrate and Delta, which genetically interacted with Notch, were found to be ligands of the Notch receptor [7–10]. Meanwhile, the homologs of *Drosophila* Notch were found in *C. elegans* (*lin-12* and *glp-1*) [11,12] and *Xenopus* (Xotch) [13], showing that Notch is evolutionarily conserved from invertebrates to vertebrates.

In 1991, a translocated membrane protein partly resembling Notch (Translocation-associated Notch homolog, TAN-1) , were found in multiple T-cell acute lymphoblastic leukemia (T-ALL) patients, suggesting the possible oncogenic role of Notch in human. This is also supported by the development of T-cell neoplasm in mice ectopically expressing TAN-1 in bone marrow progenitors [14]. It was an exciting discovery, as finding a gene directly linked to both normal development and cancer were still novel at that time. This also illustrated how the study of a development gene in *Drosophila* could have great implications in human diseases. Subsequently, the molecular mechanism of Notch signaling was explored and the respective homologs of different components of Notch were found in many metazoan species (Table 1). Notch signaling is simple in principle, but versatile in action. Notch signaling were found to be important in development, homeostasis, cancer and various diseases. From stemness maintenance to promoting differentiation, from oncogenic to tumour suppressing, Notch plays both the black and white in a context dependent manner, orchestrating the Notch Signaling Requiem, a song of birth, rebirth and death.

	Drosophila	*C. elegans*	Zebrafish	*Xenopus*	Mammals
Ligands	Delta Serrate	*lag-2*	jag1a,b deltaA,D delta-like 4	X-Serrate-1 X-Delta-1	Jagged1,2 Delta-like1,3,4
Receptors	Notch	*lin-12* *glp-3*	notch1a,b notch3	Xotch	Notch1-4
DNA-binding proteins	Suppressor of Hairless / Su(H)	*lag-1*	rbpja,b	X-Su(H)	CSL (RBPJ)
Canonical downstream targets	hairy/enhancer-of-split	*ref-1* family	her family	Esr family	Hes/Hey family, Nrarp

Table 1. Orthologs of Notch signaling

Canonical Notch signaling pathway: Simple but Elegant

Notch signaling is surprisingly simple given its long evolutionary history, dating back to cnidarian Hydra or further [15]. Its basic principle and core units are highly conserved (Fig. 1 and Fig. 2). Notch signaling is a cell-cell contact dependent signaling pathway, in which the membrane bound ligands, Jag1,2, Delta-like (Dll) 1,3,4 (mammalian homologs of *Drosophila* Serrate and Delta respectively), bind to and activate membrane bound receptors Notch1-4 (mammalian homologues of *Drosophila* Notch). Upon binding, Notch receptor undergoes a series of catalytic cleavages which lead to the liberation of the intracellular domain of Notch (NICD). NICD then translocates to the nucleus and joins the DNA-binding protein CSL (mammalian homolog of suppressor of hairless in *Drosophila*), subsequently recruits co-activators such as Mastermind-like (MAML, mammalian homolog of Mastermind in *Drosophila*) and p300, replacing the pre-occupying co-repressor and ultimately leads to the transcription of Notch target genes. Unlike many other signaling pathways, canonical Notch signaling does not involve direct amplification during signal transduction.

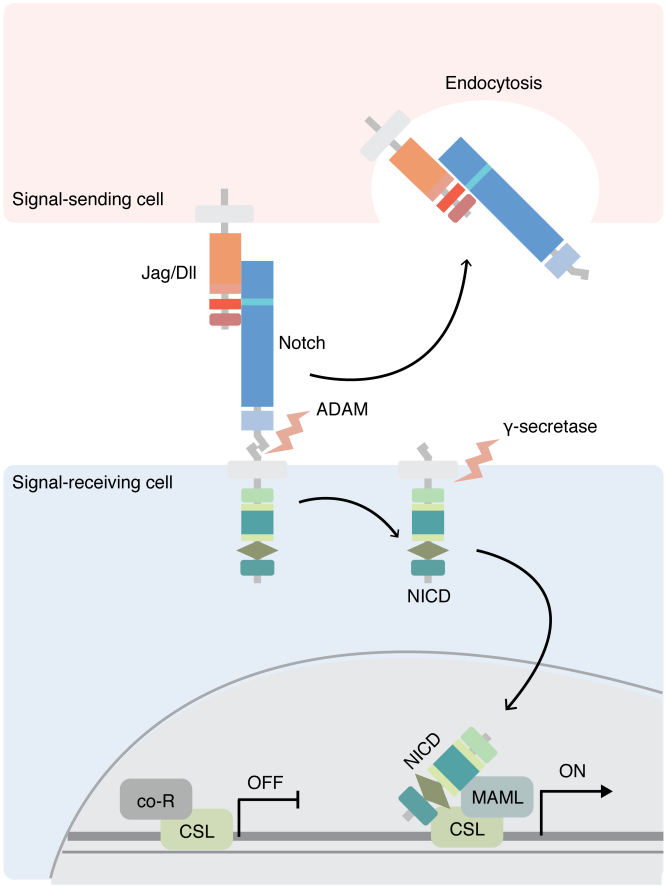


Figure 1. The canonical Notch signaling pathway. Upon binding of a Notch ligand (Jag/Dll) and a Notch receptor, a pulling force is generated by endocytosis of the ligands and the activation of Notch receptor. The Notch receptor undergoes S2 cleavage by ADAM and S3 cleavage by gamma-secretase to liberate the Notch intracellular domain (NICD). The NICD then translocates to the nucleus and forms a complex with co-activators such as MAML and the DNA binding protein CSL to switch from a Notch-OFF to Notch-ON state, activating downstream transcriptional targets.

While the general principle of Notch signaling is very simple, it is also highly versatile and most often works in a context dependent manner. How this simple mechanism could lead to complex outcomes is one of the most fascinating question in Notch signaling. Post-transcriptional modification, crosstalk with other pathways and regulation of the epigenetic landscape could be some of the ways Notch exerts its versatile actions and will be discussed further.

Notch receptors

Notch receptors are type I single-pass transmembrane proteins (Fig. 2), consist of the N-terminal extracellular domain (ECD), the transmembrane domain and the C-terminal intracellular domain (ICD). Before translocation to the cell membrane, its immature form is cleaved by furin-like convertase in the trans-Golgi at the S1 cleavage site, subsequently forming a non-covalently bonded heterodimer of the extracellular domain and the intracellular domain [16,17]. From the N-terminus, the first are the repeating EGF-like domains. The number of repeats varies among receptors and species. In mammalian Notch receptors, it ranges from 29 to 36 repeats. Repeat 11-12 are responsible for ligand interaction, as shown in binding assay and loss-of-function experiments on *Drosophila* and mammalian Notch [10,18–21]. Next follows the negative regulated region (NRR), which consists of 3 Lin-12-Notch repeats (LNR) and a heterodimerisation domain (HD). The HD is the remnant site of S1 cleavage, holding the two fragments together. The HD domain also contains the S2 cleavage site, which is accessed and cleaved by ADAM (a disintegrin and metalloproteinase) during ligand-receptor binding. The NRR is important to shield the S2 cleavage site from ligand independent activation. Mutations in the NRR compromised its inhibition and lead to auto-active Notch. This could explain why NRR is observed to be a mutation hotspot in leukemia patients[22]. Next is the transmembrane domain, which contains the S3 cleavage site. During ligand activation, S3 site is cleaved by transmembrane γ-secretase complex (γSec), subsequently releasing the NICD from the cell membrane [23].

The NICD starts with the RBPJ-associated module (RAM) and ankyrin repeats domain (ANK), which interact with the DNA binding protein CSL (CBP/RBPjk, Su(H), Lag-1) [24,25]. The ANK is flanked by nuclear localization signal (NLS). RAM plays a more important role compared to ANK in binding to CSL[26], while ANK but not RAM is required to bind to the co-activator MAML[27]. The next domain is the transactivation domain (TAD), which is only found in Notch1 and 2 but not in 3 and 4 in mammals. The C-terminus harbours a proline (P), glutamic acid (E), serine (S), and threonine (T) rich (PEST) domain, which is essential for rapid degradation of the NICD. Mutation in the PEST site would increase half-life of the NICD and thus upregulate Notch signaling [28,29].

Canonical Notch Ligands

Canonical Notch ligands are also type-1 transmembrane protein (Fig. 2), classified into two groups – homologs to *Drosophila* Serrate (mammalian Jag1,2) or Delta (mammalian Dll1,3,4) respectively. The notch ligand ECDs consist of a Module at the N-terminus of Notch Ligands (MNNL) domain, followed by a cysteine rich DSL (Delta, Serrate, Lag-2) domain, both of which are important to the activation of Notch receptors [30–32]. In a recent study, MNNL of Jag1, Jag2 and Dll4 was shown to react with the phospholipid of the cell membrane in the signal receiving cells to enhance signal transmission[33,34]. Then comes the EGF repeat domains, with the number of repeats varying among Notch ligands. The first two EGF repeats in Jag1,2 and Dll1 resemble the Delta/OSM-11 (DOS) motif in *C. elegans* and are also involved in receptor interaction[30,32]. Mutation in

the 2nd EGF repeat of Jag1 results in the loss of ability in binding to Notch1 and subsequently give rise to Alagille syndrome like symptoms in mice, as discussed in **Paper IV**. The Jag family differs from the Dll family in the presence of a cysteine rich region. Dll-3 diverges the most from the rest of the ligands, with the degenerate form of DSL domain, the lack of DOS domain, and the localization to the Golgi rather than the cell membrane, and is believed to be an inhibitor of Notch signaling [35–38].

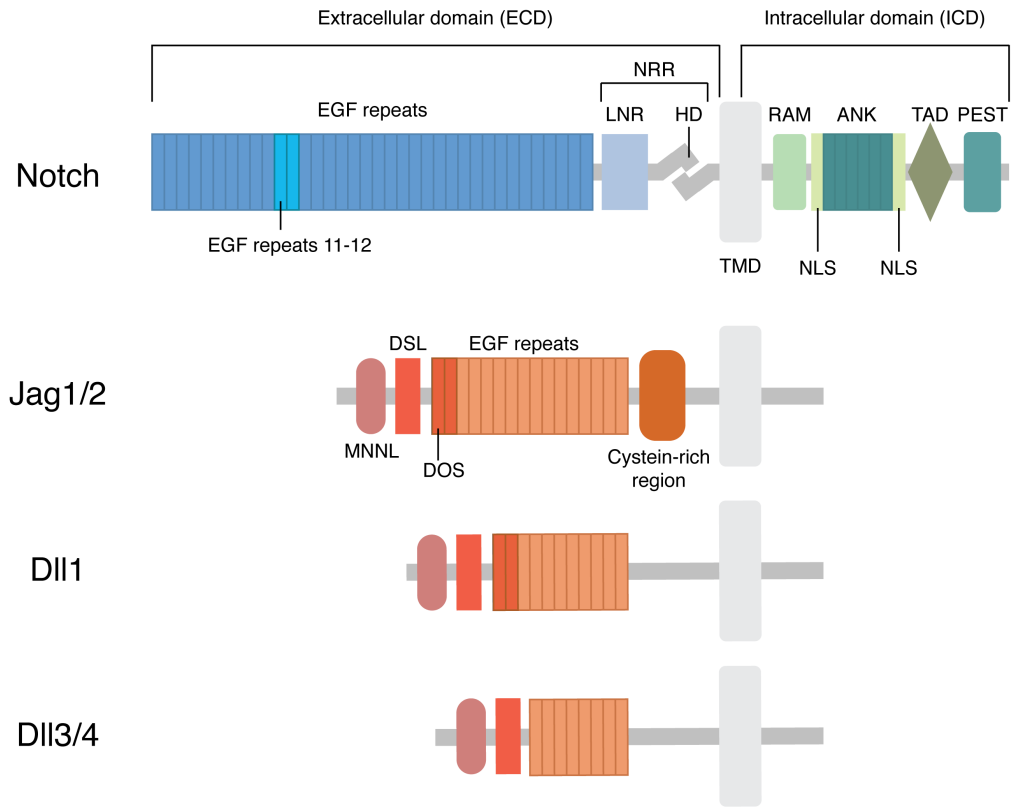


Figure 2. Canonical Notch receptors and ligands.
EGF, epidermal growth factor-like; NRR, negative regulatory region; LNR, Lin12-Notch repeats; HD, heterodimerization domain; TMD, transmembrane domain; RAM, RBP-J association module; ANK, ankyrin repeats; TAD, transactivation domain; NLS, nuclear localization sequence; PEST, proline/glutamic acid/serine/threonine-rich motifs; MNNL, Module at the N-terminus of Notch Ligands; DSL, Delta/Serrate/LAG-2; DOS, Delta and OSM-11-like proteins

Interaction of Canonical Notch Receptors and Ligands

The most well studied Notch ligand-receptor interaction is the trans-activation, where a Notch ligand from a juxtaposed signaling sending cell binds to and activates the Notch receptors on the signal receiving cell. It has been observed that Notch ligands from the same cells could inhibit the Notch receptors from receiving signal, which is known as cis-inhibition [39–41]. In trans-activation, the ligand-receptor interaction creates a mechanical force on the NECD, initiates a conformational change of NRR, which exposes the S2 site to ADAM metalloproteases mediated proteolytic cleavage. This is supported by measurement of mechanical force during interaction, and the ability of Notch induction even when the EGF domains are replaced by FKBP-FRB synthetic domains[42]. Under the

endogenous condition, this force is generated by the endocytosis of the ligands [43], together with NECD into the signal sending cell [44].

NICD and CSL

Upon Notch receptor activation, the released NICD translocates to the nucleus and forms a complex with CSL (RBPj- κ) and MAML. CSL contains three domain: NTD (N-terminal domain), BTD (β-trefoil domain) and CTD (C-terminal domain), where the NTD and CTD resemble the Rel homology region. The NTD and BTD recognize and bind to the DNA, with a weak consensus sequence C/tGTGGGAA [45]. It is believed that CSL, together with co-repressors (*i.e.* SHARP/MINT, KDM5A, and KyoT2 [46]), preoccupy Notch target sequence as a repressor of Notch targets, only until the formation of MAML-NICD-CSL complex, then switch from the repressive (Notch Off) state to the transactivation (Notch On) state [27,47]. However, there was a Chromatin immunoprecipitation (ChIP) study showing that NICD dynamically recruits CSL to the Notch targets, while CSL occupies Notch independent sites [48]. This is further demonstrated by the fact that loss-of-function in CSL does not always initiate a derepressed Notch profile [49–52]. Thus, the role of CSL as a default repressor of Notch targets is context dependent. Our results in **Paper III** are in line with the classical model, where in CSL knockout (KO) megakaryocyte (Mk) and erythroid (E) progenitor, classical Notch targets such as Hes1 and Hes5 were derepressed. Conversely, our results in **Paper I** support the later model in the breast cancer setting, as the CSL KO cell lines rarely have derepressed Notch targets. Meanwhile, majority of Notch independent genes were upregulated in the KO cells. This suggests that CSL may have a large array of actions beyond Notch signaling. A known example is its possible role as a mitotic bookmark [53]. However, the modes of Notch independent actions of CSL remains largely unknown. The non-canonical roles of CSL will be further discussed below.

Canonical Notch Targets

The outcome of Notch signaling is diverse in organisms and cell types, but there is a limited subset of conserved Notch targets that is used as a benchmark or model to study Notch activation. One is the Hes family proteins (*i.e.* Hes1, Hes2 and Hes5 in mammals), named by classical Notch targets hairy and enhancer of split in *Drosophila*. Hes is a family of bHLH transcription factors, also possessing an orange domain responsible for dimer formation, and a WRPW domain with repressive function. Hey family (*i.e.* Hey1, Hey2 and HeyL in mammals) is a subfamily of Hes that is similar to the YRPW motif. Hes factors are rapidly degraded, thus they have a short half-life [54]. On the other hand, it has been shown that Hes transcription is initiated within minutes of Notch activation [55], proposing that they may act as pulse transcriptional responders of Notch. Besides Hes/Hey, Notch-regulated ankyrin repeat protein (Nrarp) is also a common Notch targets in many instances in mammals [56]. Although these genes are the most intuitive targets for initial examination when studying Notch activation, they are

still highly context dependent and not necessarily a guaranteed benchmark of Notch activation.

Diversity in Notch Signaling

The magnitude, modes and signaling output of the Notch ligand-receptor interaction is dependent on which receptors/ligands are involved, and the modifications of the receptors and ligands. Different Notch receptors could have opposite signaling outcome in the same situation [57,58]. Notch1 and Notch3 were shown to be co-expressed in the same cell and have non-redundant functions in early intrathymic progenitor [59]. One possible explanation of the differential outcome is the variations in the NICD. For instance, the significantly shorter TAD domain in Notch3 may explain its lower transactivation activity as compared to Notch1 and Notch2 [60]. However, the selectivity cannot be fully explained by the NICD, as mice with genetically swapped Notch1 ICD and Notch2 ICD showed no significant differences in development or cancer outcome [61]. Notch4 is the least understood, as it may not be activated by ligand, but may be possible to cis-inhibit Notch1 in the same cell [62]. The discrepancy of the reaction to the two different Notch ligand families is modulated by posttranslational modification of the Notch receptors and will be discussed below. In addition, ligands in the same family, such as Dll1 and Dll4, were shown to have non redundant function in the same tissue *in vivo*[63]. The context dependent nature of Notch receptors remains largely unknown.

Posttranslational modification of Notch receptors

The ECD of Notch is modified with O-linked oligosaccharide (O-glycans). These alterations could modulate the response of Notch receptors to ligand activation. For example, O-glucose modified by Rumi is essential for Notch receptors to receive signals [64]. Modification by Fringe proteins is a typical example to show how these changes could structure and remodel the ligand-receptor interaction (Fig. 3). Fringe proteins are glycosyltransferases, first discovered in the *Drosophila* in 1994 as a modulator of Notch [65]. In mammals, there are three Fringes known as: the Lunatic fringe, Manic fringe, and Radical fringe. They function by attaching N-acetylglucoseamine (GlncNAc) to the O-fucose at the EGF repeats. These modifications by Fringe proteins play a role in regulating the response of Notch to different ligands. In mammals, Lunatic and Manic fringe enhance Delta-like trans-activation but inhibit Jag trans-activation, while Radical fringe enhances both Delta like and Jag trans-activation. It is also found that the fringe proteins have parallel effects on the cis-inhibition Notch ligands. These dynamics allow cells co-expressing Notch receptors and ligands to tweak their ability to receive and send different Notch signals [40].

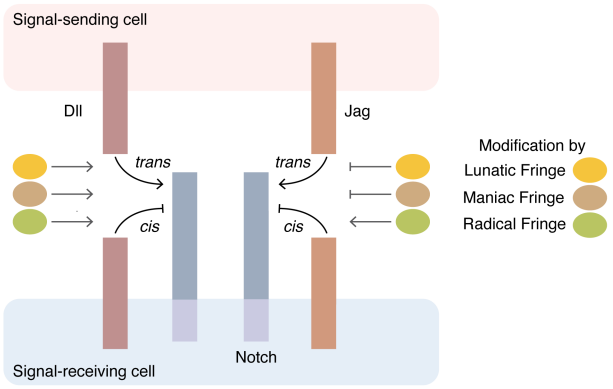


Figure 3. Modification of Notch receptor by Fringe proteins modulate trans and cis interaction of Notch signaling. All Fringe modifications enhance trans and cis interaction of Dll with Notch. Modification by Lunatic Fringe and Maniac Fringe inhibits while Radical Fringe promotes trans and cis interaction of Jag with Notch.

Modulators of Notch receptors

Besides Notch ligands expressed in the same cell and acting as cis-inhibitors of Notch receptors, there are other proteins that could inhibit Notch receptors. One classical example is Numb, which is a membrane associated protein negatively regulates Notch activity in *Drosophila*[66]. Its name comes from the loss-of-function mutation of Numb resulted in cell fate change and loss of sensory neurons. One of the proposed actions of Numb is by enhancing endocytosis of Notch, thus retaining it within the endosome [67]. On the other hand, Bardet-Biedl syndrome proteins were found to be able to promote recycling of Notch receptor from the endosome to the cell surface[68]. The endosome-lysosome transition is also modulated by proteins such as ESCRT and BLOS2, the loss of which causing accumulation of Notch receptors thereby enhancing Notch signaling [69,70]. Other than direct interaction with Notch receptors, protein kinase C (PKC) θ could also enhance Notch signaling by remodeling the actin skeleton which leads to an increase of ADAM10 recruitment [71].

Posttranslational modification of NICD

As Notch signaling does not involve an amplification step as in other signaling pathways, the dynamics of NICD plays a key role in the signaling strength and cycle. An increase in half-life of NICD is sufficient to trigger hyperactive Notch signaling and outcomes such as cancer [28]. Examples of posttranslational modification of NICD include: phosphorylation, methylation, hydroxylation, acetylation and ubiquitylation. NICD could be phosphorylated by various kinases. For example, N1ICD and N3ICD could be phosphorylated at the NLS by PIM kinases, which is important to their nuclear localization and transcriptional activity [72]. PKCζ mediated phosphorylation is important to the trafficking of the Notch receptor, as it enhances relocalization of NOTCH from the late endosome to the nucleus in Notch-ON state while it facilitates Notch internalization in Notch-OFF state[73]. Glycogen synthase kinase-3 β stabilizes N1ICD but reduces the activity of N2ICD [74]. Another kinase, CDK8, phosphorylates NICD at the PEST, which in turns promotes the PEST-dependent degradation by the Fbw7 ubiquitin ligase [75]. Nemo-like kinase (NLK) phosphorylates N1ICD near the ANK domain and decreases the trans activation activity by interfering with the formation of the active transcriptional complex. Conversely, NLK phosphorylation increases N3ICD activity [76]. Finally, a recent study identified Eya1 as a phosphatase crucial to Notch signaling. Eya

was shown to dephosphorylate N1ICD and increased its stability, which in turns led to the maintenance of Notch activity in craniofacial morphogenesis[77].

N1ICD could be methylated by CARM1 (coactivator-associated arginine methyltransferase 1) at the TAD domain after the formation of a NICD-coactivator complex. This decreases the half-life of the ICD, yet increases its signal amplitude, indicative that this methylation promotes full but short Notch signals[78]. NICD could also be hydroxylated by Factor Inhibiting Hypoxia-Inducible Factor (FIH), which will be discussed below in the crosstalk of NICD and hypoxia signaling pathway [79,80]. Notch1 ICD was stabilized by acetylation at the conserved lysine residues franking the ANK domain, and is deacetylated and destabilized by SIRT1 in endothelial cells[81]. In contrast, acetylation of the Notch3 ICD promotes proteasomal degradation and reduces Notch activity in T-ALL[82]. Ubiquitylation of NICD primes it for proteasomal degradation. The E3 ubiquitin ligase Sel-10 ubiquitylates NICD at the PEST domain and initiates its degradation[83–85]. Deltex is another E3 ubiquitin ligase that ubiquitylates NICD at the ANK domain and mediates degradation [86,87]. In recent years, there are more studies on ubiquitylation and deubquitylation as a dynamic process in the control of Notch signals. For example, the deubiquitinase Usp28 counteracts Sel-10 and causes stabilization of the NICD [88,89].

Non-canonical Notch signaling

Although the main principles of canonical Notch signaling are highly conserved, the long evolutionary history must have provided ample opportunities to develop non-canonical modes of actions. These could be categorized depending on which module of Notch signaling is altered. First is non-canonical Notch ligands that could activate Notch receptors and trigger the release of NICD; second is a CSL independent signal outcome, such as crosstalk of NICD with other signaling pathways; third is the non-canonical role of CSL, which is independent of NICD and the upstream Notch pathway.

Non-canonical Notch ligands

There have been reports of non-Jag/Dll proteins that could activate Notch receptors and elicit "canonical" downstream output. Examples include microfibrillar proteins MAGP-1 and MAGP-2, Y-box protein-1 (YB-1), Delta/Notch-like EGF related receptor (DNER) and more recently Delta-like 1 homolog (DLK1) [90–93], where most of them possess EGF-like repeats in their extracellular domain. These proteins were shown to bind to Notch receptor and cause the release of NICD and subsequent downstream signaling output. However, there is a growing realization that we still have a lot to uncover in the Notch ligand-receptor complexes [94]. The above studies did not explore the scenario where Jag/Dll ligands are absent, therefore it is difficult to conclude whether they serve as a sole ligand, or just as modifiers of canonical Notch signaling.

Non-canonical roles of CSL; Notch and epigenetics

As discussed above, the non-canonical role of CSL is largely unexplored. We have covered above the role of CSL in the crosstalk with other pathways; the recent views on the dynamic binding nature of CSL; and the vast repertoire of Notch independent CSL binding sites. Are these sites targets of CSL as in non-canonical Notch signaling? Are these sites potential Notch targets that require other co-activators or epigenetic bookmarks to be active? Are these sites completely irrelevant for signaling but relevant for the role of CSL in epigenetics? Taken together with the context dependent nature of Notch signaling, it is of particular interest to explore the role of CSL in epigenetics. As described above, CSL is hypothesized to have a role in mitotic bookmarking, as CSL remains bound to DNA during mitosis in an embryonal-carcinoma cell line [53]. In addition, CCCTC-binding factor (CTCF), a protein known to have profound functions in DNA loop formation, 3D genome organization and enhancer/promoter insulation[95], was found to directly interact with CSL and possess overlapping binding sites with CSL. Moreover, Notch signaling was found to dynamically alter the H3K4me3 signature in CSL binding sites, as Notch inhibition would cause an erase and Notch reactivation would cause a reestablishment [96]. In the same study, histone demethylase KDM5A, which erases H3K4me3 marks, was found to directly interact with CSL. These findings indicate that CSL interacts with epigenetic related proteins and play a role in epigenetic landscape. In another study, N1ICD was found to reduce H3K27me3 signature at NICD binding sites in T-ALL by antagonizing polycomb repressive complex 2 (PRC2) [97]. However, how N1ICD evicts PRC2, and whether CSL was involved, remained unexplored. There are numerous cases that Notch activation could alter epigenetic

marks, such as H3K27 acetylation in long range enhancers in T-ALL[98], and H3K56 acetylation in large amount of enhancers in *Drosophila* [99]. In **Paper I**, we showed that the ablation of CSL in the breast cancer cell line MDA-MB-231 led to a significant change in transcriptomics that is Notch independent, where individual CSL knockout clones also show subtle differences in the transcriptomics changes. Alternatively, many of the CSL KO clones cease to develop after a few passages (unpublished), implicating that CSL could have some essential function that its ablation may not be easily adapted by the cell line *in vitro*. It would not be surprising if these large scopes of changes are linked to an epigenetic role of CSL.

Crosstalk with signaling pathways

CSL independent Notch signaling has been described since the 1990s. The earliest examples were found in both *Drosophila* and mammalian Notch signaling, such as in embryonic dorsal closure, muscle cell fate in *Drosophila*, and the inhibition of muscle differentiation in mammals [100–102]. In **Paper III**, we showed that CSL-dependent canonical Notch signaling is dispensable in adult steady-state and stress myelo-erythropoiesis in mice. Taken together with the opposite results in mice with combined deletion of Notch1 and Notch2 [103], a CSL-independent Notch signaling pathway is most likely to be involved in the myelo-erythropoiesis. A more detailed look at the crosstalk of Notch signaling with other signaling pathways is performed in breast cancer cell lines, where Notch signaling was shown to upregulate interleukin-6 (IL-6) in an NICD dependent but CSL independent way, as the overexpression of dominant negative CSL did not abrogate the upregulation [104]. This study showed that NICD acts through IKKα and IKKβ from the NF-κB signaling pathway, while NICD does not need to enter the nucleus to elicit the action. Interestingly, this pathway is also independent from the canonical NF-κB signaling pathway, as it does not activate a κB reporter. This demonstrates that Notch could be versatile in terms of crosstalk with other pathways and that NICD does not always act as a co-activator. Here, I will describe some signaling pathways involved in this thesis and some to illustrate how crosstalk with other signaling pathways are mediated.

Crosstalk with the hypoxia signaling pathway

In a variety of situations (*i.e.* development, homeostasis and cancer), cells are exposed to a low oxygen environment. Even in physiological conditions, tissues are generally exposed to 2-9% oxygen content, far lower than the atmospheric oxygen level in *in vitro* culture systems most laboratories adopt. In some scenarios, the oxygen content could be extreme, where <2% is usually considered a hypoxic environment [105]. For example, in the developing embryo before placenta formation, the oxygen content could be lower than 2% [106]. Hypoxia in cancer was examined in the 20th century, as Otto Warburg observed that cancer cells prefer glycolysis rather than aerobic respiration. The radioprotective nature of highly hypoxic or anoxic (O_2 < 0.02%) environments was also reported in the early 20th century, that tumour reoccurrence was seen even after radiation in such conditions. It is only until 1990s, with the discovery of hypoxia-inducible factor (HIF), which paved the way to study the molecular mechanism of hypoxia signaling [107,108]. HIFs

are basic helix-loop-helix (bHLH) transcription factors that form a heterodimer with ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator) and binds to hypoxia response element (HRE) sequences to initiate transcription. In mammals, there are three HIFs – HIF1α, HIF2α (EPAS), and HIF3α (IPAS). Under normoxic (oxygenated) conditions, HIF undergoes oxygen-dependent hydroxylation by prolyl-hydroxylases (PHDs) and will lead to rapid degradation, while under hypoxic conditions, HIF is stabilized and can thus drive the expression of downstream genes. Years of research have proposed numerous implications of hypoxia in cancer. The reprogramming to a hypoxic metabolism has been described as one of the ten "hallmarks of cancer" [109]. In 2019, the Nobel Prize in Physiology or Medicine was awarded to Gregg Semenza, William Kaelin, and Peter Ratcliffe for their contribution to the understanding of hypoxia signaling.

The possibility of a crosstalk between Notch and hypoxia is intuitive, as cells with high population density is likely to have more juxtaposed interaction and consumption of oxygen. Hypoxia signaling could upregulate Notch by upregulation of Notch signaling components in many settings. For example, hypoxia signaling upregulates Notch1 in neuroblastoma to instigate a cell-fate change to a neural-crest like phenotype [110]. Hes1 was also upregulated, but whether it is a secondary effect of the upregulation of Notch1 or a direct crosstalk of Notch and hypoxia remained unexplored. Jag2 was found to be upregulated in breast cancer and led to an increase in vasculature formation, metastasis and cancer stem cell renewal [111,112]. Dll4 is upregulated by hypoxia signaling in vascular development and angiogenesis in cancer [113–115]. In most of these studies, Notch signaling was found to be required in the hypoxia induced response. A direct interaction of HIF1α and Notch signaling was discovered in neuronal and myogenic progenitors, such that under hypoxic conditions, HIF1α stabilizes N1ICD, enhances its transactivation activity and accompanies it to the Notch-responsive promoters [116]. Similar crosstalk is also observed in the context of cancer, where hypoxia induces migration and invasion of breast cancer cells in a Notch dependent manner, through the stabilization of NICD by HIF1α [117]. As a negative regulator of the hypoxia signaling pathway, FIH was found to hydroxylate NICD in the ANK domain and decreases its transactivation activity [79,80,118]. It has also been shown that HIF1α could directly interact with the γ-Secretase complex and enhance the γ-Secretase activity, leading to elevated Notch activity in breast cancer cell lines [119]. Taken together, these observations demonstrate that the hypoxia signaling pathway interacts with and regulates Notch signaling.

Conversely, whether Notch signaling could regulate hypoxia signaling, is less studied. Notch was speculated to directly or indirectly regulate the hypoxia signaling, as NICD overexpression could further enhance hypoxia responsive genes in mouse ES cells in hypoxic conditions [120]. In **Paper II**, we showed that Notch signaling enhances HIF2α mRNA and protein level in multiple cancer cell lines and primary cancer cells even under normoxic conditions, possibly through an intermediate effector. Interestingly, HIF1α is downregulated in some cell lines and primary cells, indicating that Notch may contribute to the HIF1α to HIF2α shift. We also showed Notch signaling requires HIF2α to

regulate a subset of Notch targets in a medulloblastoma cell line. In **Paper I**, we produced an unexpected result, i.e. that the loss of CSL led to an increase of HIF1α protein level in normoxic conditions in breast cancer cell line MDA-MB-231, through non-transcriptional control. NICD was shown to interact with HIFα, and the level of HIFα decreased when a γ-secretase inhibitor (DAPT) is applied, suggesting that NICD could stabilize HIF1α. Our two papers strongly support the notion that Notch signaling could regulate hypoxia signaling. In certain circumstances, Notch creates a "pseudo hypoxic" response, which as previously described is one of the hallmarks of cancer.

Crosstalk with the Wnt signaling pathway

Wnt signaling is another evolutionary conserved signaling pathway, important in both development and cancer settings. The name of the ligand Wnt comes from the combination of *Drosophila* gene Wingless and mammalian gene originally called Int1, as they were found to be homologous. Wnt signaling functions in a double inhibition manner. In a Wnt-Off setting, the destruction complex (containing axin APC, CK1α, and GSK3β) phosphorylates β-catenin, which is the transcriptional activator in Wnt signaling, and leads to its rapid degradation. In the Wnt-On state, the binding of Wnt to a Frizzle family receptor will disrupt the destruction complex, causing the inability of GSK3β to phosphorylate β-catenin, and thus an accumulation of β-catenin. β-catenin will then form a transcriptional complex leading to the transcription of Wnt targets [121]. Notch and Wnt work closely together in many developmental, homeostasis and cancer settings, either in synergistic, opposing, step-wise manner, or as a feedback control of one another, depending on the situation [122]. For example, Wnt and Notch play a synergistically role in cell proliferation and tumourigenesis [123], but an opposing role in stem cell identity in intestinal stem cells [124]. One mode of their interaction is transcription-dependent control. For instance, Wnt signaling upregulates Jag1 expression and thus Notch signaling in colorectal cancer [125]. Alternatively, their components could directly interact and regulate one another. GSK3β was found to be capable of phosphorylating N1ICD and decrease its proteasomal degradation in embryonic fibroblasts [74]. On the other hand, NICD could inhibit GSK3β activity in a CSL-independent manner during myogenesis in mice [126]. Whether this is due to the direct interaction of NICD with GSK3β or the secondary effect of other non-canonical Notch, was not explored. Lastly, Notch and Wnt components could interact and work together as a transactivation complex. For instance, MAML could act as a co-activator of β-catenin [95]. CSL, NICD and β-catenin were found to form a complex and activate arterial genes in vascular progenitors in mouse embryonic and adult vessels[127].

Crosstalk with the NF-κB signaling pathway

NF-κB is a family of transcription factor first identified as a DNA binding protein in B lymphocyte tumour. It is later found to be involved in several processes in immunity, inflammation and cancer. NF-κB is ubiquitously expressed but is normally inhibited by "inhibitors of NF-κB"(IκB), preventing it from translocation to the nucleus. In the active state of NF-κB signaling, IκB is phosphorylated by the IκB kinase (IKK) complex (consisting IKK α, IKKβ, and IKK γ/NEMO) leading to its rapid degradation, thus

releasing the NF-κB to translocate to the nucleus for target gene expression. In canonical NF-κB signaling, the IKK complex could be activated by Toll-like receptors (TLRs), tumour necrosis factor receptor (TNFR) and interleukin-1 receptor (IL-1R) [128]. Both Notch and NF-κB signaling were demonstrated to transcriptionally upregulate components of the other pathways, such as in immune and liver cells [129]. Meanwhile, they could also act cooperatively, such as in the regulation of the miR-223 axis in leukemia [130]. NICD was found to directly interact with NF-κB components, either activating or inhibiting them [129]. More interestingly, it was also observed that NICD and IKK could interact and act through a CSL-independent and NF-κB independent pathway [104], indicating modular mix-and-match could lead to further possibilities of signal transduction.

Crosstalk with the TGF-β/BMP signaling pathway

Bone morphological proteins (BMPs) are a group of signaling proteins discovered in 1965 as a factor with the ability to induce formation of ectopic bone structures [131,132]. They are part of the transforming growth factor β (TGF-β) superfamily (including factors such as activins, inhibins, noggin), which primarily act as ligands to the TGFβ receptors. BMPs, together with other members in the TGF-β superfamily, have profound functions beyond bone induction, including gastrulation, early embryogenesis and development of many organs. Upon binding of ligands, the TGF-β receptors, which are serine/threonine kinase receptors, form a heterodimer and lead to phosphorylation of the type I receptors in the dimer. This subsequently causes the phosphorylation of R-SMADs (named after the *C. elegans* homolog Sma and *Drospholia* homolog Mad), which could then form a heterotrimer with co-SMAD (Smad4 in mammals). The trimer then translocates to the nucleus and acts as a transcription factor to initiate target gene expression[133]. Notch signaling and TGF-β/BMP signaling could interact in a few ways. First, TGF-β signaling could regulate the expression of Notch components in various manners[134–136]. Second, SMADs were found to directly interact with NICD. For example, N1ICD was found to interact with Smad3, while cooperatively and interdependently regulate the expression of Hes1 [137]. Similar interactions were also found between N1ICD and SMAD1 [138], and between N4ICD and SMAD3 [139]. Finally, in non-canonical TGF-β signaling, TGF-β type I receptor could be cleaved by the same γ-secretase component that cleaves Notch receptors [140]. With co-immunoprecipitation, the TGF-β ICD is found to be associated with NICD, indicating new ways of how NICD could cross-talk with TGF-β signaling.

Notch in development

Developmental biology is the study of how organisms grow and develop into an organized and complex individual. It emerged post-WWI and reached a golden era over the last decades in the 20th Century. It has played a quintessential part driving advancement in cell and molecular methods, as a model to study molecular mechanisms, and inspiring other fields such as stem cell biology, regenerative biology, cancer biology and evolutionary developmental biology (evo-devo). It has also contributed to medical implications, such as developmental diseases and regenerative medicine. In the 21st Century, the focus in the field has shifted from traditional developmental biology to stem cell and regeneration, with the aim of development in relevant translational medicine. However, developmental biology is still essential. To know regeneration, one must know generation.

The study of Notch started with the end phenotype of notched wings. It stepped up a notch by the observation of its roles in embryogenesis by Poulson [4], which opened the door of genetic analysis in embryogenesis and sparkled a golden era of developmental biology. In many developmental processes, Notch signaling plays important roles in cell fate decision and maintaining stem cell identity. In certain contexts, Notch could also promote differentiation. Loss of function of Notch signaling often leads to embryonic lethality. Haploinsufficiency of Notch components often links to developmental syndrome in humans. Thus, it is imperative to understand Notch signaling during development.

Classical modes of action of Notch in development

How a single zygote could give rise to the complex organism with different patterns and cell identity has been one of the most fascinating questions in developmental biology. Alan Turing first proposed a mathematical description of how two "morphogens" with simple diffusion gradients could lead to complex biological patterns [141]. This was later demonstrated in many developmental scenarios, such as in anterior-posterior patterning and digit formation. However, patterning and cellular identity determination was not limited to diffusible morphogens only. Notch signaling was one of the classical models for studying pattern mechanisms such as lateral inhibition, asymmetric cell division and lateral induction.

Lateral inhibition

The term "lateral inhibition" was borrowed from neuroscience, where an excited neuron suppress its neighbours' activity. Similarly, in a field of cells each with an equal potential of a specific lineage, certain cells might stand out to be the "chosen ones" and suppress neighbouring cells from going towards the same lineage. This was initially described in the study of *Drosophila* sensory organ precursor (SOP) selection, where only one cell in a proneural cluster (PNC) will become SOP, creating a salt-and-pepper pattern (Fig. 4A,B,C). Notch signaling suppressed SOP determination, as loss of Notch will cause all PNC cells to adopt a SOP lineage. In the classical lateral inhibition model, SOP started as a particular cell with slightly higher proneural activity than its neighbours. This will

lead to a slightly elevated activity of Delta, which will activate Notch of its neighbouring cells, hence suppressing their proneural genes and Delta. As a result, the SOP will receive reduced Notch signal sent back from its neighbours, forming a feedback loop, further amplifying its SOP fate [142]. Recent studies suggested that this is not achieved by downregulation of Delta expression, but rather the inhibition of Neur-mediated Delta signal at a protein level [143]. This mode of action is conserved and found in other systems. For example, in mammalian angiogenesis, the tip cell of a sprout expresses Dll4 to activate Notch1/3 in neighbouring cells, inhibiting them from taking a tip cell lineage [144].

Binary cell fate decision

Asymmetric division is another classical model in Notch mediated cell fate decision (Fig. 4F). After SOP adopts its identity, it will subsequently divide into two cells with distinct lineages – one being the precursor of the sensory organ internal cells (pIIb) and the other being precursor of the sensory organ external cells (pIIa). Notch signaling promotes the pIIa lineage while suppresses the pIIb lineage, as gain-of-function of Notch leads to a pIIa lineage while loss-of-function of Notch leads to a pIIb lineage. In addition, NICD is only observed in the pIIa but not in pIIb. This asymmetry started with cell polarization, that the Notch inhibitor Numb resides to one side of the SOP. During cell division, only one daughter cell inherits the Numb, causing a Notch-Off profile and thus the pIIb lineage [67,145]. A similar mechanism is also adopted and well studied in neuroblast cell fate decision [146].

Lateral induction

Lateral induction is another classical mode of action of Notch signaling in development (Fig. 4D,E). Instead of a negative feedback loop of Notch signaling, lateral induction utilizes a positive feedback loop. The signal sending cell promotes its neighbouring cells to the same lineage of its own, meanwhile also upregulates Notch ligands in the neighbouring cells to return the same signal, forming a positive feedback loop. As lateral induction enhances the signal sent from the induced cells, it could act as a relay to pass down the signal. One example is the vascular smooth muscle cells (VSMC) in the multi-layer arterial wall. The inner-most layer of the VSMC progenitors receive Jag1 signals from the endothelial cells, upregulating their Jag1 expression and promoting its VSMC fate. With an elevated Jag1 expression, these VSMC progenitors relay the Jag1 signal to the next layer, also causing their Jag1 upregulation and VSMC determination. Thus, this second layer of VSMC progenitors could send back the Jag1 signal to the first layer, strengthening its Jag1 expression and VSMC fate, forming a positive feedback loop. At the same time, a similar loop is formed with the subsequent layer of cells [147]. Likewise, Jag1 mediated lateral induction is also observed in pancreas [148] and lens [149] development.

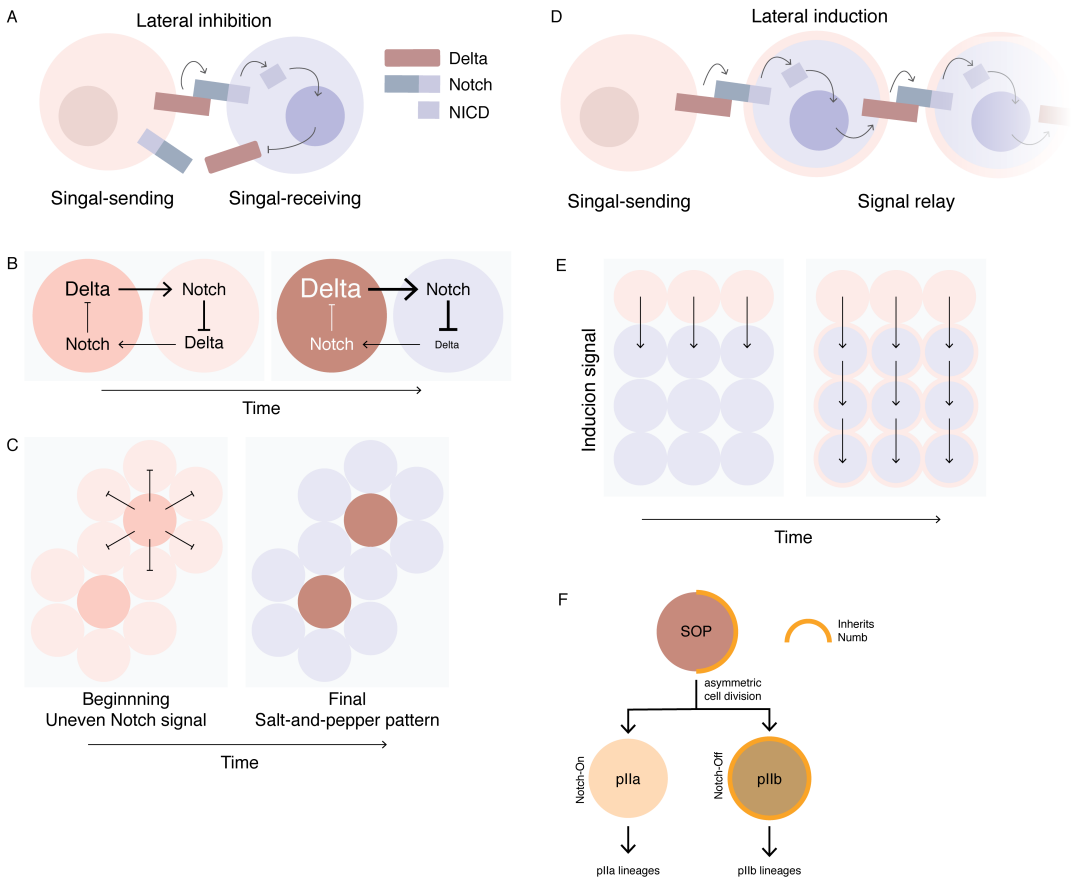


Figure 4. Classical modes of actions of Notch signaling. (A,B,C) Lateral inhibition. (A) A signal-sending cell activates Notch in an adajacent cell, suppressing the signal sent back from it. (B) The lateral inhibition feedback strengthen the identity of the signal sender. (C) Over time, an originally uneven Notch signal with lateral inhibition will lead to a salt-and-pepper pattern. (D,E) Lateral induction. (D) A signal-sending cell activates Notch in an adjacent cell, upregulating the Notch ligand in the receiving cell, allowing it to propagate Notch signal further. (E) Over time, lateral induction causes the signal and identity to pass down to subsequent layers of cells. (F) Binary cell fate decision by asymmetric cell division. An SOP undergoes asymmetric cell division, where the Notch inhibitor Numb is only inherited to one daughter cell. Thus, it generates one Notch-ON daughter cell pIIa progenitor and one Notch-OFF daughter cell pIIb progenitor, leading to distinct cell lineages.

Notch in organ and tissue development

Given the above versatile principles of the action of Notch, it is unsurprising that it plays a role in almost all organ-systems. Here, we will include some tissues and organs relevant to this thesis, and to illustrate Notch in action in various developmental processes.

Notch and the segmentation clock during somitogenesis

One of the conventional role of Notch in development is its contribution to the segmentation clock during somitogenesis. Somites are intermediate mesoderm derived embryonic structures, which give rise to ribs, muscles, vertebra and dermis, along the rostral-caudal axis in a segmented pattern[150]. Somitogenesis is the formation of somites, which starts from the caudal unsegmented growth zone in the presomitic mesoderm (PSM) of the embryo, budding one pair of new somites at the rostral end each time of a cycle. An oscillatory cycle of clock-linked genes such as *c-hairy* (chicken homolog of *Drosophila* canonical Notch target hairy) was observed. In every cycle, c-hairy is observed

transiently from the caudal side to the rostral side of the unsegmented growth zone. Upon the expression of clock-linked genes in the rostral end, cells at the prospective intersomic edge will undergo mesenchymal-to-epithelial transition (MET), forming a cleft separating the new somite and the new rostral edge of the growth zone. A recent study shows that clock is not required for the segmentation but likely to be an upstream to control its timing [151]. The Notch1, Dll1, Dll3, Lunatic fringe and Hes7 were found to be important in somitogenesis [152–156]. Inhibition of Notch signaling by GSI led to the loss of synchrony of the oscillatory clock, which is recovered after washout of the inhibitor [157]. Multiple studies confirmed that Notch signaling is important in the synchrony rather than the production of the oscillatory pattern [158–161]. This illustrates that Notch signaling could also function as a coordinator in terms of spatial and temporal synchrony.

Notch in vascular development

Notch signaling is indispensable in many stages of vascular development. Mutations of the Notch components in human often lead to vascular diseases. For example, abnormalities in the vasculature cause early onset stroke in patients with CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), which is associated with *NOTCH3* mutations. Vascular abnormalities in ALGS (refer to **"Notch in Alagille syndrome"** section) caused 34% of mortality among patients [162]. The blood vasculature originates from the lateral plate mesoderm, which first undergoes vasculogenesis to form a primitive vascular network consisting of arteries, veins and capillaries. Next, angiogenesis is the process of secondary sprouting of new vessels from this network. *Notch1*, *Notch2*, *Jag1*, and *Dll4* null mutants are all embryonic lethal in mice because of vascular abnormalities [163–166], indicative of their importance in early vasculogenesis. Notch also plays a role in arteriovenous specification. Notch activity, together with Vegf, Shh and Wnt signaling, promotes artery specification [127,167], while Notch signaling is suppressed by COUP-TFII in the veins to retain the venous identity [168,169]. Moreover, Notch also contributes to angiogenesis, by controlling the balance of specialized endothelial cells called tip cells and stalk cells. The Notch-Dll4 axis inhibits angiogenesis and tip cells formation, as ablation of Dll4 caused increased tip cells and hyperbranching [170–172]. On the other hand, *Jag1* promotes angiogenesis by antagonizing the Notch-Dll4 interaction, as the trans activation of Jag1 is inhibited by Fringe modification of the Notch receptors [173]. Notch is also important for the integrity of blood vessels, for example the vascular smooth muscles cells surrounding the vessels. Loss of Jag1 or Notch3 both led to abnormalities in the vascular smooth muscles cells [174,175]. This could potentially be the explanation behind brain hemorrhage seen in some ALGS patients and the early onset stroke in CADASIL patients.

Notch in heart development

Heart is muscle, but heart is more than muscle. The heart is grossly composed of three layers - epicardium, myocardium and endocardium, with four chambers and an outflow tract separated by septums. The heart originates from the lateral plate mesoderm and the subsequent cardiac mesoderm at E6.5 in mice. As the cardiac mesoderm (marked by

transient expression of Mesp1 at primitive streak stage) migrates near the head fold, it forms the first heart field (FHF), which moves bilaterally to form the cardiac crescent and eventually fuse to become the primitive heart tube (PHT) (E7.5 – E8.25). This progenitor pool mainly contributes to the left ventricle (LV), and partly to the atria. The second wave of the *Mesp*1^{+} cells forms the second heart field (SHF), which lies dorsally and medially to the FHF. The SHF contributes to the outflow tract (OFT), right ventricle (RV), atria and the inflow tract (IFT) [176]. As the embryo develops, the bilateral crescent folds and becomes the PHT, which opens dorsally. After its closure, the SHF joins anteriorly to form the OFT and elongate the heart tube. As the heart tube elongates, heart chambers start to balloon out and the heart tube loops to the right. The atrial and venous pole eventually moves dorsally and cranially and is thus displaced to the cranial side of the ventricles (E8.5 – E10.5). The formation of the OFT cushions (truncal cushion and conus cushion) and atrial-ventricular (AV) cushions happens at E9.5. The AV cushions fuse at the midline to separate the AV canal. The truncal cushion expands and joins the conus cushion to form a spiral aorticopulmonary septum, separating aorta and pulmonary trunk. Cardiac neural crest cells (CNCC), which originate from the ectoderm at the boundary of the neural plate, migrate and colonize the truncal cushion at around E9.5 – E10. CNCC has been found to be essential for proper septation in the OFT and remodeling for the semi-lunar valves [177,178]. The epicardium forms from the proepicardial organ (PEO), which could be marked by *Wt1* and *Tbx18*, located adjacent to the venous pole at E9.5. The PEO could contribute to interstitial fibroblast, however its contribution to the coronary vasculature and myocardium remains controversial [179].

Notch signaling plays different roles during cardiac development and has been associated with human CHD. *JAG1* was found to be mutated in 94% of ALGS patients [180,181], of which 77% were reported to have CHD. Around 15% of the *JAG1* mutated ALGS patients have Tetralogy of Fallot (TOF), a cardiac syndrome characterized by four major symptoms. Among the ALGS patients with CHD, 45% of them are TOF with pulmonary atresia (PA), a severe form of TOF. Some patients have abnormalities in both right and left heart, which is very uncommon. One case of ALGS was reported with hypoplastic left heart. However, persistent truncus arteriosus (PTA) had not been reported [182,183]. These results imply that *JAG1* may be crucial in both left and right heart development and that it may play similar roles as *TBX1*. The absence of a genotype to phenotype correlation, and that cardiac phenotype varies even within a family, is intriguing and imply there is likely to be modifiers [184]. *NOTCH2* mutations were also found in ALGS, however the percentage of patients with CHD is lower compared to the *JAG1* mutated patients in the same cohort [185]. *NOTCH1* mutations were reported to be associated with calcified aortic valve disease (CAVD) and TOF [184,186]. Homozygous *Jag1* KO is embryonic lethal in mouse, while a heterozygous mutant showed no heart phenotype [187]. The ALGS mouse model "Nodder" in **Paper IV** exhibits ASD, VSD and hypoplastic RV. Isl1$^{Cre/+}$ mediated conditional KO of *Jag1* led to VSD, DORV in the majority, and PTA in 10% of the mutants, while Isl1$^{Cre/+}$ mediated dnMAML expression lead to VSD and PTA [188,189]. However, pulmonary stenosis was only observed in a few *Jag1* conditional mice and none in dnMAML conditional overexpressed mice. This is quite different from ALGS, where PTA was not reported and pulmonary atresia appears

in more than 40% of the TOF patients. The PTA observed may be due to a gene dosage effect by the complete ablation of *Jag1* or Notch signaling in the SHF lineage, while ALGS patients only have heterozygous mutations. However, it is still of particular interest to understand why the high penetrance of pulmonary stenosis or atresia in ALGS TOF patients was not recapitulated in these mouse models, which is one of the main determinant of TOF severity in clinical perspectives. It is notable that *Isl1* was later found to be also expressed in the CNCC lineage, and that the proposed role of Notch in SHF and OFT have to be reexamined [190]. A mouse model more resembling the TOF phenotype is a *Hey2* homozygous KO mutant, where VSD, pulmonary atresia, overriding aorta and RV hypertrophy, together with a ASD phenotype, was observed [191]. Similarly, a double heterozygous mutant for Jag1 and Notch2 (*Notch2*$^{+/-}$; Jag$^{+/-}$) also displays TOF like phenotypes [188]. Moreover, Notch signaling was also found to be critical in cardiomyocyte proliferation during trabeculation and EMT in heart valve formation [192,193].

Notch in liver development

The liver originates from the foregut endoderm at E8.5 in mice, divided into two main lineages – the hepatocyte lineage and the cholangiocyte lineage. The hepatocytes are responsible for most of the metabolism in the liver, while the cholagngiocytes form the bioducts. The hepatocytes also produce bile, which is transported through the bile ducts to the gall bladder for storage [194]. In the developing liver, a layer of hepatoblasts surrounding the portal vein expresses cholangiocyte markers such as Sox9, Hnf1β, and specialize into cholangiocytes (the epithelial cells of the bile ducts), resulting in a continuous layer called the ductal plate. Afterward, some hepatocytes adjacent to the ductal plate also express cholangiocytes markers. Lumens were then formed between these bipotent cells and the ductal plate, creating the asymmetrical bile ducts. Ablation of CSL in the hepatoblast lineage reduces cholangiocyte differentiation, while activation of Notch by N1ICD expression in hepatoblast lineage promotes it [195]. Cholangiocytes express both Notch1 and Notch2, while only ablation of Notch2 impairs bile duct formation[196]. Nevertheless, simultaneous ablation of Notch1 and Notch2 in the hepatoblast lineage resulted in a stronger phenotype, indicating that Notch1 partly compensates for the function of Notch2 [197]. Heterozygous mutations of Jag1 and Notch2 lead to paucity of the bile ducts [188], revealing that the Jag1-Notch2 axis is imperative in the bile duct formation. Conditional knockout of Jag1 in hepatoblast lineage does not lead to developmental abnormalities in the bile ducts [198], denoting that the vital Jag1 signal is likely sent from elsewhere. It was later shown that Jag1 in the smooth muscle cells but not the endothelial cells is important for bile duct maturation[199]. In **Paper IV**, we established that a missense mutated Jag1 mouse line has lower Sox9 expression in cholangiocytes, and recapitulate the bile duct phenotype in ALGS patients. This is possibly due to the inability of the mutated Jag1 to bind to Notch1 and its reduced ability to activate Notch2.

Notch in haematopoiesis

Haematopoiesis refers to the generation of various cell types in blood, such as erythrocytes (red blood cells or RBC), megakaryocytes (give rise to platelets), and immune cells. They arise from two distinct haematopoietic lineages: the lymphoid lineage which gives rise to T cells, B cells and natural killers; and the myeloid lineage which gives rise to megakaryocytes, erythroid, and granulocyte-macrophage lineages. Haematopoiesis occurs at different places throughout various stages of development, and continuously occurs at the bone marrow in adult for replenishment of blood cells. The first wave of haematopoiesis is called the primitive haematopoiesis, which happens at mouse E7.5, giving rises to only erythrocytes and myeloid progenitors but not definitive haematopoietic stem cells (HSCs). The definitive haematopoiesis gives rise to definitive HSCs, capable of self-renewal and development into all HSC lineages. It begins at the aorta-gonadmesonephros regions (AGM) at E9, later shifting to the fetal liver, subsequently to the bone marrow in adults. Notch has been found to be essential in the embryonic definitive haematopoiesis in the AGM, as ablation of *Notch1*, *CSL* or Delta-Notch activating *Mindbomb* led to the loss of HSCs from AGM [200–202]. Furthermore, a recent study demonstrated that the Dll4 suppresses the recruitment of surrounding hemogenic cells in the intra-aortic hematopoietic cluster [203]. Meanwhile, primitive haematopoiesis seems to be Notch independent, as the loss of Notch does not lead to alterations in the haematopoietic progenitors from such process [200]. Notch signaling is important in the T-cell/B-cell switch in the lymphoid lineage. Activation of Notch1 signaling skews differentiation towards the T-cell lineage instead of the B-cell lineage [204], while inactivation of Notch1 or CSL inhibits T-cell development but promotes B-cell development [205,206]. The roles of Notch signaling in the erythroid and megakaryocytes lineages are highly debated. Some studies showed that Notch-Dll1 favors the megakaryocytes lineage over the erythroid lineage [207], while other studies showing the opposite, *i.e.* that Notch signaling favors the erythroid lineage but suppresses the megakaryocyte differentiation [103,208]. CSL null mouse embryos exhibited reduced apoptosis of yolk sac originated erythroid cells [209]. In **Paper III**, we showed that canonical Notch signaling is dispensable in adult myelo-erythropoiesis by haematopoetic specific ablation of CSL. This indicates that the results in other studies, which used different experimental strategies including perturbation of Notch receptors and ligands, may be driven by non-canonical Notch signaling, which is worth reexamination.

Notch in Diseases

A brief history of cancer

Cancer is the malignant form of development, the uncontrolled growth of invasive and even metastatic tissues. Fossil evidence showed it might have presented in human ancestors as early as 1 millions years ago [210]. In modern human, cancer has been known for a long time and described by Egyptians as early as 3000 BC [211]. The word cancer came from the Greek word "karkinos" (crabs), which was used by Hippocratic physicians to describe tumours, as they were often compared to the shape of a crab [212]. The long history of cancer does not prevent it from being a mysterious disease before the knowledge of mutation and oncogenes. In late 19th and early 20th Century, different factors were thought to be linked to cancer, ranging from chimney sweeping, virus, to radiation. Radiotherapy and chemotherapy were used to treat cancer, yet many cancer patients reacted differently and some were even insensitive to treatment. Relapse in cancer is often met with grave prognosis and was described as early as in the 1st Century [211]. With modern genetics and molecular biology knowledge, we now know cancer is not a single disease, but a collection of diseases. Different mutations can cause different subtypes of cancer, and mutations could progress through time. These mutations could lead to oncogenes, mutated genes that could potentially cause cancer, or disruption of tumour suppressor genes, genes that are vital in protection from cancer development. Even within a tumour, there is a high degree of heterogeneity. Hanahan and Weinberg described ten checkpoints cancer development has to go through as hallmarks of cancer [109]. As mentioned above, many oncogenes were found to be signaling-related. Among them, Notch signaling is one on hot pursuit. Therefore, it is imperative to investigate the role of Notch signaling in cancer to fully understand the underlying mechanism and develop relevant treatment.

Notch as an oncogene

The study of Notch in humans started with its association to cancer, as the truncated and translocated form of Notch1 (TAN-1) was first cloned in a few T-ALL patients [213]. Jon Aster's group later confirmed the connection from the discovery of frequent gain-of-function mutations in Notch1 in T-ALL patients (54 out of 96) [28]. These mutations are mostly located either in the NRR region, leading to disruption of the protection from γ-secretase, or in the PEST domain, leading to an increased half-life of the NICD. The gain-of-function nature of these mutations was confirmed experimentally in the same study. The first evidence of the oncogenic role of Notch in solid tumours came from the study of how mouse mammary tumour virus (MMTV) induced breast cancer in mice, where one of the insertion by the virus was later found to trigger the expression of N4ICD [214,215]. To date, Notch signaling is known to be correlated with and have implications for many different types of cancer, such as breast cancer, medulloblastoma, colorectal cancer and non–small cell lung carcinoma [216,217]. Notch signaling could directly promote cell proliferation in cancer. For instance, the Jag1-Notch1/3 axis was found to directly upregulate cyclin D1 expression and subsequent cell cycle progression in human triple negative breast cancer cell lines and in rat cell lines [218,219]. Promotion of cell

proliferation or survival was also reported in many cancers, such as adrenocortical carcinoma [220], glioma [221] and liver cancer [222]. Additionally, Notch1 was observed to have anti-apoptotic activity in prostate cancer [223]. Besides cell survival, Notch activity plays a role in metastasis and recurrence. Blockade of Notch signaling could inhibit EMT and subsequent metastasis in human breast cancer xenograft in mice, by rescuing the low E-cadherin expression via Slug suppression [224]. It has been shown that hypoxia enhanced cancer migration and invasion is dependent on Notch signaling. Moreover, overexpression of NICD in normoxia could replace hypoxia in induction of cell invasion [117]. There are also reports of correlation of high Jag1 expression to metastasis and recurrence in prostate cancer [225,226], which merits further studies.

Notch as a tumour suppressor

As described above, the outcome of Notch signaling is highly context-dependent. Although mutations in Notch were originally discovered to be oncogenic, and found to be hyperactive in many cancers, the role of Notch mutations as a tumour suppressor was also reported. Notch signaling typically acts as the stem cell gatekeeper in many organs, which could explain why hyperactive Notch could lead to tumourigenesis. However, Notch signaling has an opposite role in skin, *i.e.* it promotes differentiation and cell cycle arrest rather than stem cell identity [227–230]. It is not surprising that loss-of-function of Notch is oncogenic in such settings. In many squamous cell carcinoma (SCC), recurrent mutations of Notch signaling components were found, such as in head and neck SCC, cutaneous SCC, lung SCC, oesophageal SCC and bladder SCC [231–235]. Most of these mutations reside in functionally important domains, thus likely to be loss-of-function mutations. Loss of Notch1 facilitated chemically induced skin cancer in mice, possibly through increased Shh and Wnt signaling [236]. Notch inactivation by ablation of CSL or expression of dnMAML in mice promotes bladder SCC progression [237]. Expression of dnMAML also result in perturbed suppression of oesophageal cell population with carcinogenic mutations in p53, which could be an early event in oesophageal SCC development [238]. Besides SCC, Notch signaling was also revealed to have tumour suppressing activity in forebrain tumour subtypes, as the inactivation of Notch through ablation of CSL enhanced glioma tumour growth and promote neuroectodermal-like tumours in the absence of p53. However, in these studies, Notch inactivation alone does not seem to directly induce cancer, but rather enhanced the progression of developed cancer or cancer induction by other agents.

Notch in breast cancer

As of 2018, breast cancer has been the most commonly diagnosed cancer, and is the leading cause of cancer related death in over 100 countries [239]. Breast cancer arises from the mammary system, which is responsible for milk production and secretion for the nurture of offspring. Unlike many other organs, morphogenesis of the mammary system predominantly occurs at postnatal period, puberty, and undergoes cycle of development and involution during pregnancy. The mammary system consists of lobular units, which are the primary units for milk secretion, connected by collecting ducts converging at the nipple. The lobules and ducts are made of a basal membrane, a myoepithelial layer, an

inner luminal layer, and a central lumen. During mammary development, the mammary stem cells activate from a quiescent state, and could specialize to become either basal progenitors or luminal progenitors. The basal progenitors will form the myoepithelial layer, while the luminal progenitors will develop into two subtypes, either double positive progenitors of estrogen receptor (ER) and progesterone receptor (PR), or the ER^- PR^- progenitors. The former will form the luminal cells, while the latter will form the alveolar cells (mammary gland development is reviewed in [240]). Breast cancer arises from the lobular units, and can be classified into multiple subtypes, including the Luminal A (ER^+, PR^+, $HER2^-$), Luminal B (ER^+, PR^+, $HER2^+$), $HER2^+$ (ER^-, PR^-, $HER2^+$), Basal-like (ER^-, PR^-, $HER2^-$) and Claudin-low. These could be corresponding to origins from mammary progenitors at different stages and lineages. In clinical settings, breast cancer with no ER, PR or HER2 markers is diagnosed as triple negative breast cancer (TNBC). Basal-like, Claudin-low and TNBC have the worst prognosis, and frequently develop resistance to chemotherapy [241].

As mentioned above, breast cancer is the first solid tumour that was found to be linked to Notch signaling, by MMTV causing N1ICD and N4ICD expression and inducing mammary tumours [215,242]. This is confirmed by luminal specific overexpression of N4ICD, where mammary tumours develop after transgene activation [243]. In **Paper V**, we found that expression of N1ICD in luminal lineage after lactation is sufficient to cause mammary tumour, although in lower frequency compared to the MMTV induced ones. Although not as commonly found as in T-ALL, mutations of Notch receptors were found in breast cancer patients [244,245]. High levels of *NOTCH1* and *JAG1* expression have been shown to correlate to poor survival in breast cancer patients [246]. In addition, NUMB mediated regulation of Notch was found to be lost in ~50% of breast cancer in a study, and the level of NUMB is inversely correlated with tumour grade [247]. Accumulating evidence has implicated the important role of Notch signaling in the metastasis of breast cancer. Inhibition of Notch signaling by the expression of N4ECD inhibited EMT and metastasis in xenograft of human breast cancer cells in mice, as the Jag1-Notch axis upregulates endogenous Slug, which then downregulates E-cadherin [224]. Additionally, hypoxia-enhanced cell migration and invasion requires Notch signaling, where overexpression of NICD could replace hypoxia to induce such increase in migration and invasion in normoxic conditions [117]. Notch signaling is also important in the glycolytic switch of metabolism, another hallmark of cancer, in a breast cancer xenograft model [248]. Moreover, Notch could elicit resistance in breast cancer therapy [249]. Besides canonical Notch signaling, non-canonical Notch signaling also plays a role in breast cancer, as the mammary tumours caused by N4ICD expression [243] were later found to be CSL independent [250]. In **Paper I**, we found that ablation of CSL is pro-proliferation, anti-apoptotic, pro-angiogenic and leads to higher homogeneity in xenograft of human breast cancer cells in mice, and that the transcriptomic change is largely distinct from canonical Notch signaling. This indicates that non-canonical Notch signaling could play an unexpected role in breast cancer.

Notch and Alagille syndrome

Alagille syndrome (ALGS) is a rare autosomal dominant multisystem disorder, found in 1 in 30,000 infants. It was initially described by French pediatrician Daniel Alagille in 1969. The symptoms include the easiest identified cholestasis (caused by liver and bile duct abnormalities), followed with abnormalities in eyes, skeleton, facial muscle, kidney, and the heart, although different symptoms are variable in penetrance [251]. Mutation mapping and sequencing results revealed the predominantly mutated gene in ALGS patients to be *JAG1*, which is found mutated in 94% of ALGS patients [180,181], while *NOTCH2* mutations were found in only 1% of ALGS patients [185]. Liver problems, including paucity in the bile ducts, are the most severe and visible phenotypes, as it causes cholestasis and subsequently jaundice. Liver abnormalities are found in more than 95% of patients, where 15% of patients develop liver cirrhosis and failure, which require liver transplantation. Congenital heart disease is also commonly found in ALGS patients (77% to >90%) [251]. Two-thirds of them have pulmonary stenosis and 15% of them have TOF, which is a severe form of heart defects with ventricular septal defect, aorta misalignment and possibly pulmonary stenosis and even atresia, causing difficulties to provide oxygenated blood to the body. These complex heart problems contribute to early mortality. Vascular abnormalities are prevalent in ALGS and contribute to 34% of deaths in one study [162].

There have been numerous attempts to generate mouse models for ALGS, however most displayed limited phenotypes. For example, three different heterozygous Jag1 mutant mouse lines have the inner ear phenotype, *i.e.,* the Headturner [252] , Slalom [253] and Ozzy [254] (all named by their behavior due to inner ear problem). Conditional ablation of *Jag1* also help understanding the role of Jag1-Notch axis in individual organ-system affected in ALGS [199,255,256], however may not reflect the haploinsufficient nature of *Jag1* mutations in ALGS. A double heterozygous mouse line for J*ag1* and *Notch2* reflects symptoms in multiple organ system as in the ALGS patients, including abnormalities in bile duct differentiation, kidney, heart and growth, although *NOTCH2* mutations are rarely found in ALGS patients [188]. A more recent study found that the heterozygous null mutation of Jag1 in C57B6 genetic background leads to a bile duct phenotype, advancing the understanding of the Jag1-Notch axis in bile duct development [257]. Although these may not fully recapitulate all the ALGS phenotypes, they are valuable for the understanding of the role of Jag1 in development of different organ system. In **Paper IV**, we established a homozygous Jag1 missense mutant mouse line "Nodder", which is able to reflect phenotypes in most organ system affected in ALGS.

Methods in Notch signaling

Molecular biologists have always been the pioneers in invention and engineering in biological sciences. Much of today's knowledge and tools in modern biology, biotechnology and pharmaceuticals came from pivotal advances in molecular biology, ranging from molecular cloning, recombinant DNA technology, tissue culture, cross-species xenografts, antibody-based tools to gene editing tools. I will here provide a brief overview of the methods used and developed to study Notch signaling.

Activation and inhibition of Notch

One of the best tools in the arsenal of Notch signaling regulation is γ-secretase inhibitors (GSIs), such as DAPT. GSI inhibits γ-secretase from cleaving the Notch receptor, thus blocking all NICD mediated responses in cells. Inhibition of canonical Notch signaling could also be achieved by overexpression of dominant negative (dnMAML), dominant negative (CSL), or truncated Notch ECD as a competitive inhibitor (genetic tools will be discussed below).

Conversely, activation of Notch can be attained by immobilized Notch ligands, as soluble Notch ligands have been shown unable to activate Notch signaling. In a typical *in vitro* setting, protein G is first coated on a cell culture dish, followed by IgG fragment (Fc) conjugated Notch ligand coated on the plate. It is also worth to note that upon cell dissociation by trypsin, Notch receptors will be sheared and Notch targets will be activated. This should be considered in experimental design. Overexpression of NICD is also a commonly used method. Additionally, we utilized a NICD-ER^{T2} fusion protein expression vector, where NICD only enters the nucleus upon the presence of tamoxifen (genetic tools will be discussed below). Similarly, co-culturing of cells expressing Notch ligands is another option. In some cases, Notch ligands are expressed in cells from one species while Notch receptors are expressed from another. With the S3-cross-specific-sequcing method our lab have developed, one could specifically analyze transcriptomic results from either the sender or the receiver [258]. Moreover, agonistic and antagonistic anti-Notch antibodies is also available as a tool to activate or inhibit Notch signaling[259].

Genetic tools: expression vectors, recombinant DNA, reporters

Overexpression of genes is one of the most direct tool in molecular biology. It is usually achieved by transient expression of plasmid or stable integration. Modified RNA (ModRNA) is an emerging clinically relevant tool, as it is risk free of random integration, and the short expression time window could be ideal for therapy or temporal studies [260]. Typically, overexpression of a transgene is driven under a ubiquitous promoter such as: CAG, UBC, EF1α (for mammalian expression). EF1α is one of the shortest mammalian ubiquitous promoter, however one should note that it may not work in all mammalian cells. Inducible expression is often achieved with the doxycycline inducible Teton system (required an expression of Tet transactivator). To express more than one transgene under the same promoter, a 2A peptide sequence or an internal ribosomal entry site (IRES) are often used. The 2A peptide sequences are peptides susceptible for spontaneous cleavage, therefore enable two transgenes to split into individual proteins after translation. On the

other hand, IRES provides an alternative site for the ribosome to start translation, thus two transgenes are translated separately. For stable integration, lentivirus is commonly used. However, since it has a cargo size limit of 4kb, and as cDNAs of many Notch components are larger than 4kb, lentivirus may not be optimal. We utilized a Piggybac transposase system, which is highly efficient, with a cargo size of up to 100kb [261]. The integrated transgene could be silenced over time, therefore, knock-in to the AAV locus or ROSA26 locus is preferable for stable expression. With CRISPR Cas9 technology, it is considerably more feasible to achieve this with limited efforts [262,263](See "**CRISPR Cas9 Gene-editing**"). Alternatively, one could introduce genetic insulators flanking the transgene to avoid silencing [264]. The Cre-recombinase loxP system is also frequently used as a conditional knockout or lineage activation tool. Upon expression of Cre recombinase, the DNA sequence flanked by two loxP site (floxed) will be removed. In **Paper V**, we used a ROSA26-floxed-StopCassette-NICD-IRES-EGFP mouse line, in which NICD and EGFP will be expressed in the entire cell lineage once Cre is present. An effective stop cassette typically contains a few poly A signals, to ensure the mRNA is polyadenylated before the transgene.

Besides overexpression of full length Notch receptors or Notch ligands, overexpression of NICD is a commonly used strategy for gain-of-function studies. Conversely, overexpression of dnMAML is often utilized to inhibit canonical Notch signaling. However in **Paper III**, our results with CSL ablation differed from those in previous studies using dnMAML, indicating that dnMAML may affect pathways other than Notch signaling. The use of dnMAML thus has to be carefully reexamined. Fusion protein is an effective technique to add or remove functions from a protein, for example adding florescent tags for easy visualization and possibility of live cell imaging or fluorescence resonance energy transfer (FRET) experiments to study protein-protein interaction. A V5 tag or FLAG-tag is ideal for immunoprecipitation (IP) or ChIP, because of their short length and availability of high-quality antibody. These tags are easily introduced by CRISPR to the endogenous locus, making it easy to study the biology of the endogenous protein, avoiding artifacts introduced by overexpression. In **Paper II**, we used a N1ICD ER^{T2} (NERT2) fusion protein, where NICD is prevented from translocating to the nucleus except in the presence of tamoxifen. Lastly, 12xCSL reporter is a plasmid with 12 CSL binding sites preceding a reporter gene, serving as a reporter of Notch signaling. We have integrated this into a Piggybac plasmid, making it easy to generate Notch reporter cell lines or mice.

CRISPR Cas9 Gene-editing

Although powerful tools such as transgenic, overexpression and gene knockdown methods exist, the gene-editing approach stands out in a few ways. For example, gene-editing enables complete KO of a gene, providing a better alternative to siRNA knockdown approaches, which may still has remnant expression of the target gene. Furthermore, precise gene-editing reflects a more relevant genotype in human diseases (*i.e.* point-mutation). In introducing a tissue specific expression of a transgene, it is easier to knock-in the transgene of interest to an endogenous locus, compared to using an

exogenous promoter, which requires previous knowledge in the first place, but may not yield accurate tissue specificity. Traditionally, gene-editing relied heavily on homologous recombination, which occurs with extremely low probability. In addition, it also requires cloning of large donor constructs and a series of selections, rendering the process time-consuming and tedious. Recent advances include protein-based targeting tools such as zinc finger nuclease and TALEN [265], which still requires substantial cloning effort. Therefore, it is revolutionary in gene-editing when CRISPR(clustered regularly interspaced short palindromic repeats)-Cas9, an RNA-guided gene-editing tool, emerged [266].

CRISPR-Cas9 is a reverse-engineered tool based on the immune system in bacteria *Streptococcus pyogenes.* In brief, the bacteria uses a Cas9 endonuclease and guide RNAs (gRNAs) to identify and remove viral inserts. Following reverse engineering, the CRISPR-Cas9 tool boils down to two main components—the Cas9 endonuclease protein, and a single gRNA (sgRNA) with a custom recognition sequence. The most commonly used *S. pyogenes* Cas9 (SpCas9), together with the presence of an sgRNA with a 20bp custom recognition sequence, targets matching sites at the genome, provided that the site precedes a PAM(protospacer adjacent motif) sequence of "NGG". The Cas9 endonuclease will then create a double stranded break three bp 5' from the PAM sequence. This will trigger the cell to undergo DNA repair with non-homologous end joining (NHEJ), which in turn randomly deletes or inserts short sequences at the break, potentially causing a frame shift mutation. In addition, if a DNA construct is also provided, it may be inserted to the break site. Furthermore, if two double stranded breaks are made, large deletion may also occur [267], providing an alternative way to achieve gene knockout. Alternatively, if a donor construct with homology arms (each greater than 50bp) is provided, the cell may undergo homology directed repair (HDR) and seamlessly replaced the disrupted sequence with the donor. As the sgRNA could be easily designed and produced within a few days, CRISPR-Cas9 is an easy and versatile tool. Its high efficiency make *in vivo* genome editing viable, both facilitating genetically engineered cells and animals, and the possibility of gene-editing based therapy. To date, advances are made by introducing other members of the CRISPR-Cas family, or by engineering the Cas9 endonuclease. For instance, Cas9 alternatives with different PAM recognition sites, Cas9 alternatives with smaller molecular size, Cas9 nickase, Cas9-based activator, RNA-editing CRISPR and single base pair substitution tools have been developed [268].

In **Paper I**, we successfully used CRISPR-Cas9 to knock out CSL in breast cancer cell line MDA-MB-231, creating a more refined version of the loss-of-function experiment as compared to an shRNA based CSL knockdown study. We also knocked out CSL, Notch1 and Notch2 in the medulloblastoma cell line DAOY in **Paper II** with the same method. Meanwhile, we developed a Cas9 based lineage tracing system (CAST) (unpublished), where Stop cassettes are flanked by a combination of different gRNA target sites. Thus, the transgene could be activated by a combination of gRNA

expression, allowing a logic-gate like control. The general design principles and recommendation for CRISPR-Cas9 gene-editing experiments are summarized in Table 2.

	Design principles/ Recommendation
Introduction of Cas9 protein	**DNA vector**: Cas9 expression by Px458 (GFP selection) or Px459 (puromycin selection) from Addgene. **Cas9 mRNA**: Better for single-cell injection to avoid mosaicism.
Introduction of gRNA	**DNA vector :** gRNA sequence cloned into Px all-in-one vectors. **Synthesized double stranded DNA:** Gblock (IDT) with T7 promoter and terminator. ***In vitro* transcribed gRNA**
Design of gRNA	**Tools:** CHOPCHOP v3 [269,270] (more sgRNA design and prediction tools are reviewed by Liu *et al.*[153]). **Criteria:** Preceding a PAM sequence; high predicted efficiency and low predicted off-target score [269,270]. **Remarks**: Cell types and epigenetics may severely affect the efficiency of the cutting [271] even with high predicted score.
Benchmarking and troubleshooting of gRNA	**Remarks:** The efficiency of the gRNA seems to be the determinant in most CRISPR experiments. Therefore benchmarking and troubleshooting of the gRNA is essential. **Methods:** T7 endonuclease assay; or PCR followed by TA cloning and sequencing. **Troubleshooting:** Repeat with different gRNAs. If no efficient sgRNA is found in a particular locus, it is recommended to try large deletion (0.5-3kb) approach or a large fragment knock-in approach, using sgRNA targeting sites distal from the original locus.
Screening of positive cell lines	**Remarks:** The most time and labor intensive part. **Method 1:** Manual picking of single cell colony. Cleanest but the most intensive method. **Method 2:** Introduction of a small DNA fragment with positive selection markers. Less demanding and easier for bulk production.
Design of knock-in construct	**Distance of homology arms from cut site**: Closer to 15bp and no further than 50bp from the sgRNA target site [272]. **Size of homology arms**: Recommended to be >800bp [272], although we also experienced success when knocking-in a 1kb construct with each homology arm of 100bp (data not shown). Study has shown that the efficiency of CRISPR-Cas9 mediated HDR with large insert increases as the length of the homology arm increases, while decreases as the insert size increases [273]. ***In vivo* generation of knock-in animals:** With a homology arms of length 1.5kb/1kb, we successfully knock-in a 2.5kb IRES-

	$CreER^{T2}$ construct to the mouse Jag1 locus to generate an F0 mouse with pronuclear injection (data not shown), indicating a 1:1 ratio is a practical range for CRISPR based transgenic animal generation. **Remarks:** Newer studies have shown that *in vivo* linearization of the homology donor by targeting the plasmid at both ends of the homology arms could drastically reduce the sizes of the homology arms to 25-100bp for large inserts, and up to 1kb-5kb in multiple systems [274–276], thus greatly reducing the cloning effort needed for homologous recombination

Table 2. Design principles and recommendation for the design and implementation of CRISPR experiments.

Transcriptomics

Transcriptomics is the study of gene expression at the individual gene level. It is a powerful tool to interpret the activity of individual genes and to provide insights into spatial and temporal transcriptomic differences in biological processes, and functional perturbation in loss-of-function or gain-of-function conditions. Combined with functional enrichment analysis such as gene ontology (GO) and gene set enrichment analysis (GSEA), one could gain new insights in biological processes. Furthermore, in combinations with other sequencing methods such as chromatin immunoprecipitation (ChIP) sequencing (ChIP-seq), assay for transposase-accessible chromatin using sequencing (ATAC-seq) and bisulphide-sequencing, one could explore the relationship between the transcriptomes and DNA-binding proteins, chromatin structure, or epigenetic status. Transcriptomics has been extensively used to study Notch signaling, as many of its responses are transcriptional. As CSL is a DNA binding protein, transcriptomics facilitates our genome-wide understanding of its role in transcription and gene regulation. In **Paper I**, we used RNA sequencing (RNA-seq) to identify Notch targets and differentially expressed genes (DEG) in CSL KO breast cancer cells, and we discovered that many DEG in CSL are not Notch targets. In **Paper II**, the transcriptomics analysis enabled us to isolate Notch targets that are HIF2a dependent. In **Paper IV**, bulk sequencing results of ALGS mouse models and ALGS patients led to the discovery of the functional relevance of the DEG and a common DEG across species. Taken together, it demonstrated that RNA-seq is a powerful tool.

The first generation of transcriptomics was the micro-array, based on hybridization of the oligonucleotides on chips. However, this is limited to the previous knowledge of known transcripts, and is subjected to high noise due to cross-hybridization. The next generation sequencing (NGS) is revolutionary, especially for genomics and transcriptomics. There are many methods of next generation RNA-seq, but in principle all function by capturing and reverse transcribing the sequences, breaking them down into small sequences and separating individual sequences, pre-amplification, and fluorescent detection during elongation or ligation. After the sequencing and quality control of these small reads, they

will be aligned to a known genome, thus allowing identification and quantity calling. Likewise, *de novo* transcriptomes could be assembled without prior genomic knowledge.

R and Python are two popular scripting/programming languages for RNA-seq downstream analysis and bioinformatics analysis (compared in Table 3). R[277] is a scripting language created by statisticians, with its focus on statistics and data science. The advantage of R is its built-in tools and syntax for statistics, and a large repository of bioinformatics packages built by bioinformaticians (many accessible through bioconductor [278]). For instance, the graph plotting package ggplot2, although comes with a steep learning curve, is often the go-to solution for most graph plotting tasks. Furthermore, RStudio is a free integrated development environment (IDE) for R, making R scripting and project management user-friendly. For most biologists with no computer science background, R with RStudio is the easiest plug-and-play tool for bioinformatics. However, programmers may not found themselves at home with R, as its syntax is significantly different from most programming languages. Moreover, it is not trivial to plug R scripts as a component of other applications. Still, R is definitely the most straightforward and useful language for biologists.

In contrast, Python is a versatile scripting/programming language widely used in software development. It is a flexible language and is easier for debugging and maintaining consistency than R. It is also easier to write data science tools with python to facilitate communication with non-bioinformaticians collaborators. Similar to R, there is a large repository of data science and bioinformatics packages in python. However, there is a steep learning curve to get python working for data science. The easiest is to get the Anaconda distribution version of python, where a lot of data science packages were maintained. PyCharm is a recommended free IDE for python, and could also be downloaded with Anaconda in one-go.

The most common analysis of RNA-seq is to identify differentially expressed genes (DEG), *i.e.* genes that are upregulated or downregulated when compared to samples. DESeq2 and edgeR are two popular R packages for DEG, using similar statistical methods and assumptions to calculate DEG. The frequentist approach [e] adopted to test DEG should be viewed with caution, as there will be a considerable amount of false positives when thousands of genes were tested (Bayesian and frequentist statistics in science was briefly reviewed by Puga *et al.* [279]). The default solution in these packages is using Benjamini-Hochberg adjustment to create an adjusted *p*-value and a false discovery rate (FDR). Meanwhile, packages using a Bayesian approach such as MMSeq, BitSeq and

[e] Frequentist statistics refers to the statistical inference framework based on observation of frequency of the data. It is traditionally used by scientists, featuring well-established methods such as statistical hypothesis testing and confidence intervals. Its main alternative, Bayesian statistics, instead used new information to update the probability for a hypothesis, featuring concepts such as prior an posterior probability and likelihood ratio.

ShrinkBayes are emerging. However, the higher computational demand and the difficulty to introduce Bayesian concepts to the biology community make the usage of Bayesian methods not as popular as frequentist methods. Gene enrichment analysis such as GO, GSEA, and tools like PANTHER and spring are helpful in enlightening us the functional meaning of the upregulated or downregulated genes. Similarly, the data could be visualized with dimension reduction methods such as principle component analysis (PCA), to see how different the transcriptomes are and what genes contribute to these differences.

	R	Python
Nature	Scripting language specialized for data science and statistics	A scripting or programming language for all-round development
Ease to use	An easy plug-and-play tool for biologist	Not as straight forward for biologists. Difficult to setup for data science. (*i.e.* maintaining versions, preparing interpreters and creating virtual environments.)
Versatility	Inconvenient to integrate to other apps	Easy to integrate to other apps
Coding styles/ features	Procedural-code; No coding style convention; Unconventional codes (count from 1 instead of 0, weird datatypes); Non-standard evaluation	Object-oriented programming; Generally accepted coding style convention.
IDE	RStudio	PyCharm: good for analysis and app development Jupyter notebook: good for testing ideas and sharing codes
Repository/Distribution	Bioconductor	Anacoda, miniconda, pip
Popular data science/bioinformatics packages	dplyr: data frame processing; ggplot2: graph plotting; edgeR/DESEQ2: RNA-seq analysis; Seurat: scRNA-seq	numpy: scientific computing; pandas: data frame processing; seaborn: graph plotting; scanpy: scRNA-seq

Table 3. A comparison of R and Python for biologists

Single cell RNA seq

Single cell RNA-seq (scRNA-seq) is the RNA-seq at single-cell level. The emergence of scRNA-seq was another huge leap in the transcriptomics field, as there are a lot of hidden information among the heterogeneity of a cell population. Single cell transcriptomics can reveal heterogeneity, common and rare cell populations, and lineage relationship among cells and population. There are two main camps of RNA-seq technique, one is based on full length sequencing (*e.g.* Smart-Seq2 [280]), the other is 3'end based with unique molecular identifiers (UMI) (*e.g.* Drop-seq [281]). The former provides a higher sequencing depth while retaining the sequence of the full transcripts, making it ideal for allelic specific analysis, splicing analysis and *de novo* transcriptomics analysis. The latter has the capability to process much higher number of cells, but at the expense of lower numbers of captured and sequenced transcripts. This higher width but lower depth nevertheless gives strong gross analysis and statistical power (for more detailed comparison, please refer to [282,283]). Because of higher variation, more noises and zero-inflation in scRNA-seq results, the typical methods for bulk sequencing may not be directly applied. Many scRNA-seq specific packages emerged, such as Seurat (R)[284], Monocle (R)[285], RaceID (R)[286] and scanpy (python; now also provide methods to integrate Seurat into python)[287], with different normalization and testing methods. Cell cycle scoring[288] is introduced to remove variation caused by cell cycle differences, however should be carefully applied as cell cycle differences could also have biological meaning. PCA is normally not enough to break down the greater heterogeneity in large scRNA-seq data, therefore machine learning dimension reduction methods such as tSNE [289] (T-distributed Stochastic Neighbour Embedding) and UMAP [290] (Uniform Manifold Approximation and Projection) were widely used in scRNA-seq analysis, with the latter more popular as it is superior in illustrating global distances among clusters.

Present Investigations

Aims

This thesis aims to investigate Notch signaling with a modular approach: how different modules in Notch signaling contributes to cancer and development, and its interaction with other pathways in the process.

- Canonical and non-canonical role of CSL in breast cancer.
- Crosstalk of NICD with hypoxia signaling.
- Role of CSL in adult steady-state and stress myelo-erythropoiesis.
- Role of Jag1 in Alagille syndrome

Paper I

CSL is the central node of canonical Notch signaling, as the whole family of Notch receptors relies on the binding of CSL to the DNA. Ablation of CSL or using dominant negative CSL is a way to abrogate Notch signaling in various studies. However, the Notch independent role of CSL was less considered. Here, we demonstrated that ablation of CSL could lead to a large change of transcriptome, and that a majority of these changes are Notch independent. The loss of CSL in the breast cancer cell line MDA-MB-231 caused increase tumour growth, decreased apoptosis and enhanced angiogenesis in xenograft. We also found the loss of CSL led to a hypoxic response in normoxic condition.

To study the role of CSL in breast cancer, we used CRISPR-Cas9 to generate a CSL null breast cancer cell line MDA-MB-231. We designed a sgRNA targeting exon 5 of CSL, and introduced it to the cells together with Cas9 endonuclease. Screening by western blot for CSL, we were able to generate multiple clones of the CSL KO cell line. Interestingly, we also observed a decrease in mRNA level by RT-PCR, indicating that there may be nonsense-mediated mRNA decay in the process. It is noteworthy that a significant number of these clones were lost after a few passages, hinting to a drastic change in the transcriptome. Despite showing signs of difficulties to be maintained *in vitro*, CSL null cell lines promote tumour growth both in xenograft model and in chorioallantoic membrane (CAM) tumour model (confirmed by two individual clones). Using Ki67 staining, and cleaved caspase 3 staining, we observed increased proliferation and decreased apoptosis in the CSL null xenograft tumour compared to the $CSL^{+/+}$ xenograft tumour. We also noted an increase of angiogenesis in the CSL null xenograft tumour, as displayed by increased vascularization stained by endothelial markers collagen IV and CD31. Matrigel invasion assay results illustrated an increase in invasion of the CSL null cell line. Taken together, the loss of CSL promotes breast cancer tumour growth and invasion. This is in line with the enhanced tumourigenesis observed by knocking down CSL in another study, and that 33% of invasive breast cancer from the Cancer Genome Atlas (TCGA) data has genomic loss in CSL [291].

We noticed a hypoxic response in the CSL null cell line in normoxic conditions, as shown by the stabilization of HIF1α, which is normally degraded under normoxia. There were no differences in the mRNA level of HIF1α, indicating that it is a post-transcriptional event. The effect is reversed by the reintroduction of CSL to the CSL null cell line. Hypoxia responsive genes such as VEGF-A, STC2 and KLF8 were elevated in the CSL null clones, although with variability among the clones. Angiogenesis, which is often enhanced in hypoxia conditions, was increased in the CSL null xenograft tumour. Next, we explored the molecular mechanism of the hypoxic response. The reducing agent DTT lowered HIF1α in CSL null cells, suggesting the stabilization of HIF1α due to nitrosylation of HIF1α or destabilization of the ODD domain. We also uncovered that N1ICD interacted directly with HIF1α in the CSL null cells. Interestingly, DAPT (a GSI) decreased the HIF1α protein level in the CSL null clones in both normoxic and

hypoxic conditions. This raises the possibility of N1ICD playing a role in the stabilization of HIF1 α in a non-canonical way. First, as CSL is already abrogated, and as our transcriptomic data have shown, canonical Notch targets were unresponsive in the CSL null cell line. Therefore, DAPT should not affect the canonical Notch targets in the CSL null cell lines. Moreover, only 11 genes were still responsive to Jag1 in the CSL null cell lines when compared to the control (unpublished), denoting there are limited non-canonical Notch targets affected by DAPT in the CSL null cell line. Together with the fact that DAPT treatment decreases HIF1α level, it is therefore reasonable to hypothesize that N1ICD could stabilize HIF1α.

The CSL null cell lines also exhibited a polyploid giant cancer cell (PGCC) like phenotype when cultured *in vitro*. The PGCC is reported to have cancer stem cell like properties and could be induced by hypoxia or $CoCl_2$ induced pseudo-hypoxic conditions. In the CSL null cell line, this could be induced by the increase in HIF1α protein. Through live imaging, we observed a defect in mitosis in a considerable portion of the cells. As CSL has been shown to bind to DNA throughout mitosis [53], it may play a role in mitosis and mitotic bookmarking.

Furthermore, we examined the transcriptomic changes in the CSL null cell line. and found a substantial change in the transcriptome with over 1700 DEGs in the CSL null cell line compared to the $CSL^{+/+}$ cell line. Among them, only 47 of 139 Notch targets were derepressed, indicating that CSL is not by default inhibitory when Notch is inactive. Kulic *et al.* [291] argued that the tumourigenesis enhanced by loss of CSL is due to the derepression of 170 "Notch signature" genes. However, we only observed 5 of such genes derepressed in the CSL null cell line. Nevertheless, derepression of such a subset of Notch target genes could play a role in increased tumourigenesis. Our results from the large transcriptomic changes and the hypoxic response indicate that the Notch independent role of CSL should not be disregarded.

In conclusion, we demonstrated that the loss of CSL enhanced tumourigenesis and unleashed a hypoxic response in normoxic conditions, accompanied by hypoxic related phenomena such as increased angiogenesis and PGCC. We showed that Notch signaling could modulate hypoxia signaling, and can have implications for tumour development. We also showed a largely canonical-Notch-independent role of CSL, and that the non-canonical role of CSL is also important in tumourigenesis.

Paper II

It is known that hypoxia can modulate Notch signaling and sometimes requires Notch signaling for regulation of its downstream targets (See "Cross-talk with hypoxia signaling" section). It is less explored whether Notch signaling could modulate hypoxia signaling. In keeping with Paper I, here we showed that Notch signaling can upregulate hypoxia signaling in various cancer cell lines. Notch activation promotes transcription of HIF2α indirectly, and contributes to a HIF1α to HIF2α shift in cancer. We also found that a portion of Notch targets are HIF2α dependent. All these findings provide evidence that Notch signaling can modulate hypoxia signaling, and requires hypoxia signaling.

In paper II, we first observed from publicly available data that HIF2α mRNA level was increased in Notch activated conditions but decreased when Notch signaling was blocked. We corroborate this by expressing N1ICD in 9 cancer cell lines, where 8 out of 9 cell lines exhibited an increase in HIF2α mRNA in the presence of N1ICD. This was also reflected in a more physiologically relevant experimental design of Notch activation with immobilized Notch ligands in the breast cancer cell line MDA-MB-231 and primary breast cancer cells. Both immobilized Jag1 and Dll4 increased the HIF2α mRNA level, which is abrogated by DAPT. This increase of mRNA level by overexpression NICD was also observed in primary glioblastoma cells and tumourigenic primary mesenchymal cells. Together, we showed that Notch can induce HIF2α mRNA level in various types of cancer.

As NICD could function in non-canonical ways (*i.e.* do not involve nuclear localization), we next examined whether the increase in HIF2α mRNA requires nuclear localization. In the medulloblastoma cell line DAOY, we transiently expressed a NICD-ER^{T2} fusion protein (NERT2), where the NICD is blocked by ER^{T2} from entering the nucleus, unless tamoxifen is present. Expression of NERT2 in the absence of tamoxifen did not lead to increase in the mRNA level of known Notch target Nrarp, nor HIF2α. On the other hand, expression of NERT2 with tamoxifen led to an increase in mRNA levels of Nrarp and HIF2α. This increase was abrogated by the expression of dnMAML or the ablation of CSL. These data confirm that canonical Notch signaling is involved in the regulation of HIF2α mRNA level.

We next investigated whether canonical Notch signaling directly regulates HIF2α expression. The ChIP sequencing results did not reveal any CSL binding sites in the HIF2α promoter region. Moreover, activation of Notch signaling using immobilized Jag1 did not increase the luciferase activity from HIF2 promoter reporter assay. These data suggest that canonical CSL does not directly regulate HIF2α expression. Translational blockade by cycloheximide (CHX) also obliterated the increase of HIF2α mRNA level by Notch activation. This suggests that canonical Notch signaling most probably upregulates the expression of an intermediate protein, which is essential for the increase in HIF2α mRNA level.

Protein stabilization and degradation are vital parts of the regulation of HIF protein levels. In normoxic conditions, HIF proteins are constantly degraded. Therefore, we examined whether the HIF2α protein is also upregulated by Notch. We observed an increase in the HIF2α protein level by overexpression of N1ICD in multiple cell lines (primary breast cancer cells, human medulloblastoma cell lines D324 and DAOY, VHL-deficient 786-O renal carcinoma cell line) even in normoxic conditions. In contrast, ligand stimulation only led to an increase in HIF2α protein level in the hypoxic conditions but not in normoxic conditions. Furthermore, HIF1α protein levels were decreased by Notch activation in some cell types, notably at later time points after Notch activation. This suggests that Notch activation may contribute to the HIF1α to HIF2α transition, which has been reported to promote stem cell characteristics and aggressiveness in cancer [292].

We then explored the functional aspect of the regulation of HIF2α by Notch activation. HIF2α targets (*i.e.* VEGF and AREG) were upregulated by Notch activation. To further understand how the interplay affected the transcriptomic outcome, we activated Notch by tamoxifen expression in a DAOY cell line stably expressing NERT. Meanwhile, we knocked down HIF1α or HIF2α by siRNA and performed transcriptomic analysis. Our data revealed that 21% of the Notch targets required HIF2α, while only 4.1 % required HIF1α. Gene enrichment analysis showed that these genes are related to GO terms in cell adhesion, blood vessel development, and signal transduction. Lastly, we found that N2ICD but not N1ICD expression enhanced tumour growth in CAM model of DAOY cell line. To further understand the different roles of Notch1 and Notch2, we used CRISPR/Cas9 to knock out Notch1 or Notch2 in DAOY cells. Ablation of Notch2 but not Notch1 dampened the tumour growth in CAM assay, in line with the NICD overexpression experiment. RNA-seq revealed that only a portion of DEG is common among Notch1$^{-/-}$ and Notch2$^{-/-}$ cell lines, indicating that Notch1 and Notch2 serve different roles in DAOY cells. To our surprise, ablation of HIF2α enhanced tumour growth in the CAM assay. This could be due to the increased HIF1α protein level observed in the HIF2α$^{-/-}$ cells. Further investigation are needed to understand how the dynamics of Notch1/2 and HIF1/2α could contribute to tumour growth.

To conclude, we identified that canonical Notch signaling could indirectly upregulate HIF2α in a broad range of cancer cell types. We also established that a subset of Notch signaling requires HIF2α. We discovered that Notch could contribute to the HIF1α-to-HIF2α switch, which is a topic that should be researched further. Collectively, these data are important to understand the role of Notch and its crosstalk with hypoxia signaling in cancer, and would provide insights for future Notch and hypoxia signaling based treatments.

Paper III

While the role of Notch signaling in embryonic hematopoiesis and lymphoid lineage has been well-studied, its role in adult bone marrow myelopoiesis is still controversial. Studies on Notch signaling in myelopoiesis are limited and not always consistent [103,207,208]. Most of these studies perturbed either the Notch receptors or the Notch ligands, therefore these results could not rule out the role of non-canonical Notch signaling. To understand the role of canonical Notch signaling in adult myelo-erythropoiesis, we specifically ablated CSL in the adult myeloid lineage by crossing either Mx1-Cre or Vav-Cre mice with homozygously loxP site flanked (floxed) CSL mice ($CSL^{fl/fl}$). We revealed that canonical Notch signaling is dispensable in adult steady-state and stress myelo-erythropoiesis, and that myelo-erythropoiesis are not impaired when CSL is ablated.

In **Paper III**, we first examined the adult myelo-erythropoiesis in the CSL ablated mice. No defects were observed in distinct stages of granulocyte-macrophage (GM), erythroid (E), or megakaryocyte (Mk) progenitors, as shown in FACS analysis. Similarly, GM, E and MK colonies, circulating platelet and red blood cells (RBC) counts showed no differences in the CSL ablated mice. Chimeric experiments of wild-type CSL cells and CSL ablated cells exhibited no differences in terms of contribution to various progenitor lineages, replenishment of myeloid cells, nor platelets in peripheral blood. Phenylhydrazine(PHZ)-induced hemolytic anemia experiments revealed differences in the RBC reduction, nor expansion of E progenitors, showing that CSL ablation caused no defect in the erythropoiesis response to stress. Lastly, quantitative analysis of Notch targets demonstrated derepression of *Hes1* and *Hes5* by CSL ablation in Mk progenitors, pre-CFU-Es, and CFU-Es, indicating that CSL may act as a repressor of these Notch targets.

Our results revealed differences compared to other studies using other approach to inhibit Notch signaling [103,207,208,293]. The most obvious explanation could be that non-canonical Notch signaling is involved in those experimental settings, that either the Notch receptor or ligands were perturbed. A previous study demonstrated that yolk sac derived erythroid cells has reduced apoptosis in CSL null mouse embryos. However, the role of CSL in erythroid apoptosis is restricted to the embryonic erythroid cells, as the CSL null embryos die at E10.5 [209]. Some of the other studies used the deletion of Nicastrin, which may have an impact on signaling mechanisms other than Notch. Lastly, our results differ from the study using dnMAML[208] is not well understood, but it is plausible that it relates to dnMAML's effect on other pathways. Therefore, studies using dnMAML to inhibit Notch signaling have to be carefully re-interpreted. In sum, we showed that canonical Notch signaling is dispensable in adult steady-state and stress myelo-erythropoiesis.

Paper IV

Alagille syndrome (ALGS) is a rare multisystem disorder mainly caused by *JAG1* loss-of-function mutation. The autosomal dominant nature of inheritance and later genetic mapping showed that ALGS relevant *JAG1* mutations are haploinsufficient. There have been many attempts to generate mouse models for ALGS, however most have limited phenotypes displayed, or do not accurately mimic the human disease (See "**Notch and Alagille syndrome**"). Here, we established a homozygous Jag1 mutant mouse line "Nodder", which is able to recapitulate relevant phenotypes in most ALGS affected organ system. We explored the transcriptomic changes in the liver due to Jag1 mutation, and found that IGF1 is a commonly affected gene in mouse and humans (although in humans, the control samples were derived from patients with other liver diseases). Lastly, we examined the molecular mechanism and showed that Jag1 with Nodder mutation failed to bind to Notch1, while weakening the ability to activate Notch2.

In **Paper IV**, we characterized our previously described "Nodder" mice, which possess a H268Q mutation in the second EGF repeat domain in Jag1, a region found mutated in a portion of ALGS patients. The heterozygous mice exhibited a head-nodding phenotype, thus were called "Nodder" (here we refer the mutated Jag1 as $Jag1^{Ndr}$). In a C3H pure background, $Jag1^{Ndr/Ndr}$ is embryonically lethal [294], however when bred into a C3H/C67Bl6 background, it reached a recovery rate of 10% at postnatal day 0 and 5% in adulthood, consequently enabled us to investigate symptoms more clinically relevant in human ALGS. We observed impaired growth, jaundice, ASD and VSD, iris deformation and craniofacial abnormalities, presenting wide range of symptoms relevant to ALGS in human.

We next assessed one of the most common and critical symptoms of ALGS – cholestasis and impaired liver function, which is related to paucity of bile ducts. $Jag1^{Ndr/Ndr}$ mice displayed jaundice, a possible result of the bile duct defect. Histological examination demonstrated that $Jag1^{Ndr/Ndr}$ E18.5 embryos lacked Sox9 and Hnf1β (early cholangiocyte markers) expression in the cells surrounding the portal vein. At a later stage, at P0, only a few cells with low Sox9 expression were observed, as compared to the control which are already undergoing lumen formation. By P10, no bile ducts were found in the $Jag1^{Ndr/Ndr}$ pups, while the wild type mice displayed mature bile ducts. In contrast, lumenized bile ducts could be located in $Jag1^{Ndr/Ndr}$, however with the majority abnormal and few well-formed. Remarkably, $Jag1^{Ndr/Ndr}$ pups at P10 showed Sox9 expression close to the portal veins, indicating that at later stage, $Jag1^{Ndr/Ndr}$ could recovered some Sox9 expression. Together with the results that liver organoids from $Jag1^{Ndr/Ndr}$ have normal cholangiocyte differentiation marker expression, we validated that $Jag1^{Ndr/Ndr}$ causes delayed but it does not completely inhibit differentiation. However, $Jag1^{Ndr/Ndr}$ organoids collapse more often than the $Jag1^{+/+}$ organoids, demonstrating structural instability.

We compared the transcriptomes of liver samples from ALGS patients to those from non-cholestatic patients, and transcriptomes of liver samples from $Jag1^{Ndr/Ndr}$ to those

from $Jag1^{+/+}$. GSEA analysis revealed common gene sets enriched in either ALGS or $Jag1^{Ndr/Ndr}$ samples, including DNA repair, E2F targets, G2M checkpoint and reactive oxygen species pathway. DEG analysis revealed 16 commonly upregulated and 2 commonly downregulated genes in both the ALGS and the $Jag1^{Ndr/Ndr}$ samples. Although the comparison is not on par, as the control sample for the ALGS samples are not from healthy patients but from autoimmune hepatitis patients (due to ethical reason), these genes may provide insights into the biology and treatment of ALGS. The commonly downregulated genes include IGF1, which was confirmed in $Jag1^{Ndr/Ndr}$ mice with ELISA. It is in line with the observation that IGF1 levels in ALGS patients are not responsive to growth hormone [295].

Lastly, with co-culture experiment with human cells expressing Jag1/$Jag1^{Ndr}$ and mouse cells expressing Notch receptors, we determined that $Jag1^{Ndr}$ lost the ability to bind to Notch1 but not Notch2 and Notch3. Even $Jag1^{Ndr}$ can bind to Notch2 and Notch3, but it exhibited a lower ability to internalize Notch2 or Notch3 ECD, while also contributing to less Notch activation of Notch2 and Notch3 as shown in a 12xCSL reporter assay.

Collectively, we have established and characterized an ALGS mouse model "Nodder". We showed that our mouse model faithfully recapitulates symptoms in multiple system seen in ALGS patients. We also revealed that the $Jag1^{Ndr}$ causes a delayed in differentiation and morphological defects in bile duct formation, possibly through the decrease in its ability to activate Notch2. We demonstrated clinical relevance by in parallel analyzing $Jag1^{Ndr}$ and ALGS patient samples. These data inform our understanding of ALGS, and provide a new model to study and develop therapy for ALGS.

Paper V

Hyperactive Notch signaling has been linked to breast cancer (See **"Notch in Breast Cancer"**), however how hyperactive Notch contributes to tumourigenesis is not fully understood. Virus driven NICD expression, such as in MMTV-N4ICD and MMTV-N1ICD transgenic mice, which are active in the mammary gland prior to birth [296], led to mammary tumour development, though the specific roles in luminal cells were not well explored [215,297]. Whey acidic protein (WAP) is a protein secreted in the milk. It is highly induced during pregnancy and lactation, but ceases to express after weaning [298–300]. Therefore, the WAP promoter is useful to induce expression in the mammary luminal cells during lactation. WAP promoter driving N4ICD expression led to a moderate phenotype, where ductal growth was not developed but lobular differentiation was inhibited [301]. However, in that report WAP-Cre lineage tracing showed that the tumour growth in the WAP-N4ICD mice were not from the WAP lineage, arguing that hyperactive Notch played a role in the stromal environment but not the tumour itself. It is worth noting that WAP expression mostly regresses after lactation. However, WAP-Cre lineage tracing revealed that remnant cell lineage of WAP expressing cells still exist after ductal tree regression (designated parity identified mammary epithelial cells, *i.e.,* PI-MECs)[302]. These cells possess stem cell properties and are capable of becoming both luminal and myoepithelial lineage. In our study, we revisited this question but using an alternative transgenic approach.

In **Paper V**, we explored the role of hyperactive Notch1 in the WAP lineage, therefore not restricting only to cells that transiently express WAP, but also to cells derived from the original WAP-expressing cells. This was achieved by crossing a WAP promoter driven Cre recombinase (WAP-Cre) mouse line with a ROSA26-loxP-stop-loxP-N1ICD-IRES-EGFP (R26-N1ICD) mouse. Upon Cre expression, the floxed stop cassette will be removed in the genome, leading to the expression of N1ICD and EGFP, causing N1ICD expression and EGFP marking in the WAP cell lineage. As a control, we crossed the WAP-Cre with ROSA26-loxP-stop-loxP-tdTomato mice. We showed that EGFP or tdTomato were expressed only after pregnancy and exclusively in luminal lineage.

WAP-Cre;R26-N1CID mice exhibited a drastically impaired offspring survival, where only 60% of pups survived after birth in the first pregnancy, but no offspring survived during the subsequent rounds (2^{nd}, 3^{rd}, 4^{th}) of pregnancy. The offspring however survived with foster mothers, indicative that it is a lactation problem. Three-dimensional morphological analysis were performed with iDISCO, displaying unaffected ductal tree in virgin WAP-Cre;R26-N1CID mice, but significantly increased in branching and number of nodes after first round of lactation. This structural defect in ductal tree formation, led to the inability to nurture newborn pups.

We next examined whether the hyperactive Notch1 in WAP lineage caused mammary tumours. We observed mammary tumours in two out of five WAP-Cre;R26-N1CID females, 26 weeks after lactation, whereas no tumours were observed in the WAP-

Cre;R26-tdTomato control mice. Surprisingly, the majority of tumours were EGFP positive, an unusual conclusion compared to the data from the WAP-N4ICD;WAP-Cre;R26-lacZ mice used in a previous study, where no lacZ cells were found in the tumours [303]. In one of the mice that developed mammary tumours, we observed metastases to the lung. The metastases were also EGFP positive, indicating that they originated from the WAP lineage. The contrasting result compared with the previous study could be explained by the different duration of NICD expression. The WAP-Cre;R26-N1CID mice exhibits N1ICD expression in the entire WAP lineage, even after regression of endogenous WAP expression, while in the WAP-N4ICD mice [303], expression of N4ICD was non-continuous, only occurring after each round of lactation.

Lastly, we explored the heterogeneity and the transcriptomic differences in the WAP-Cre;R26-N1CID and WAP-Cre;R26-tdTomato mice by single cell RNA-seq of EGFP or tdTomato sorted cells. Dimension reduction with PCA and UMAP and unsupervised clustering revealed that there were six subtypes of cells identified from both mouse lines. In some subtypes, the distribution of N1CID-EGFP or tdTomato cells were even, while in others, either N1CID-EGFP cells or tdTomato cells were enriched. Gene enrichment analysis revealed that immune system related GO terms were enriched in the N1CID-EGFP biased subtypes. On the other hand, GO terms of metabolism and catabolic processes were enriched in one of the tdTomato enriched subtypes, while GO terms of cell adhesion is enriched in another one of such subtypes. Further studies are required to understand the gene expression differences and how they may contribute to the tumour formation.

Jointly, we showed that hyperactive Notch in WAP lineage could lead to ductal formation defect and mammary tumour. We also demonstrated that WAP hyperactive Notch lineage compose mammary tumour and subsequent metastases, in contrast to a previous study. Lastly, we established that the heterogeneity among the luminal cells was perturbed by Notch activation, however more efforts are required to fully understand how this imbalance may lead to tumourigenesis.

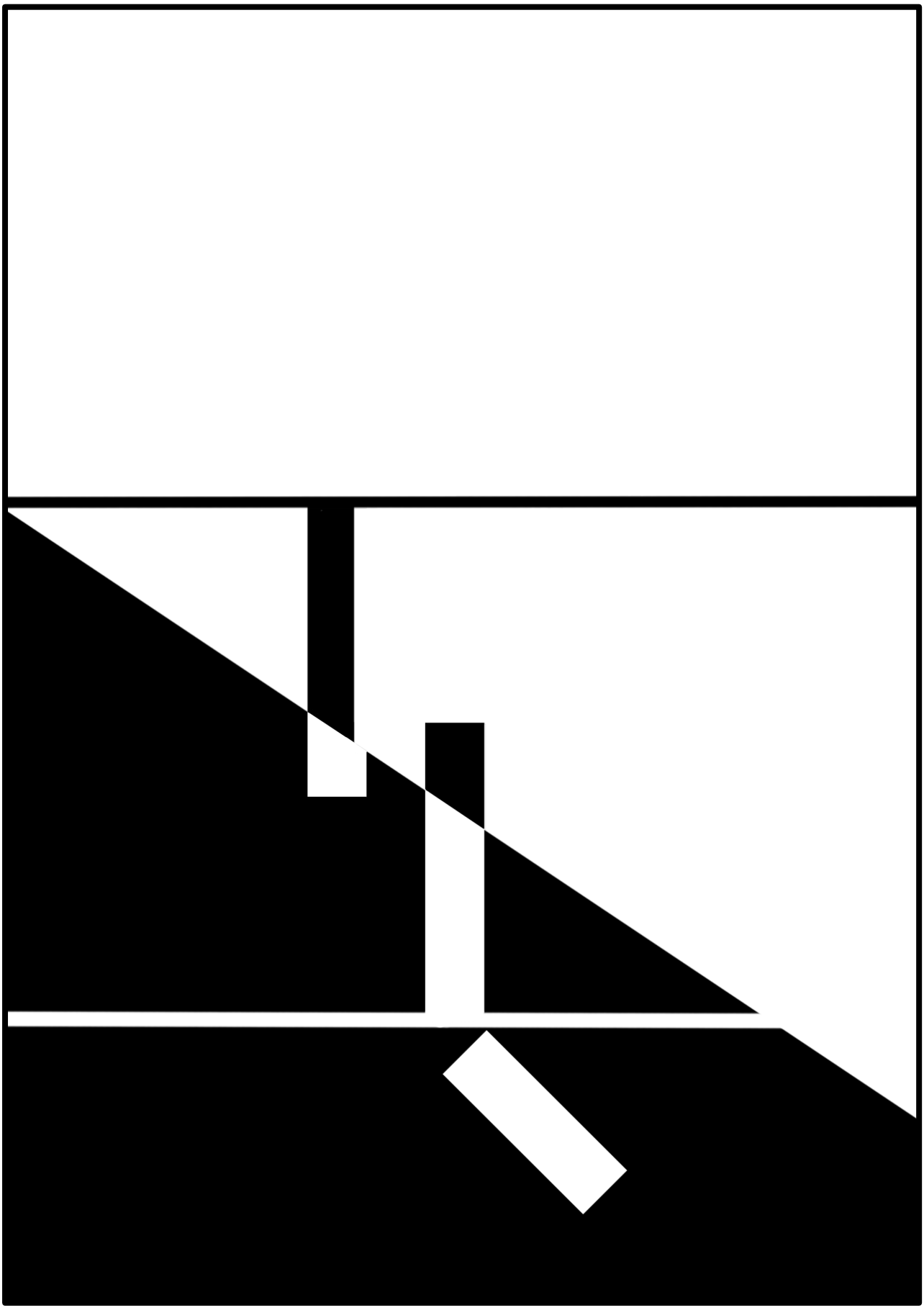
Future Perspectives

It is intriguing that **Paper I** and **Paper II** showed that both canonical and non-canonical Notch signaling can modulate hypoxia signaling. However, we have not yet elucidated the direct molecular mechanism, for example how the loss of CSL lead to upregulation of HIF1α. To address this, one could reintroduce a defective CSL (*i.e.* with mutated DNA binding domain), to test whether the observed effects are related to direct transcription of an intermediate protein that exerts the effect. Second, we speculated that NICD could stabilize HIF1α in this "pseudo-hypoxic" response induced by CSL ablation. This could be further investigated by overexpression of NICD in the CSL null cells. Lastly, the large Notch-independent transcriptomic changes in the CSL null cells remain unexplored. We uncovered a limited number of derepressed Notch targets in the CSL null cells. ChIP sequencing of CSL and other epigenetic markers could elucidate the dynamic binding of CSL in such contexts, and its roles in epigenetic regulation in breast cancer. In **Paper II**, we revealed that Notch upregulates HIF2α mRNA via an intermediate protein. ChIP sequencing of NICD and CSL, and cross comparison with the transcriptomic data may shed light on the identity of the intermediate protein. Similarly, we observed differences by expressing either N1ICD or N2ICD, or by ablating Notch1 or Notch2. It is a suitable model to study how the two Notch receptor paralogs in the same cell would serve different function, remains an unanswered question in Notch signaling. As in **Paper III**, we observed different results of the CSL ablated cells with overexpression of dnMAML in myelopoiesis, indicating that the commonly used dnMAML may not fully represent removal of canonical Notch signaling, but may also have other effects. For instance, MAML has been shown to be a co-activator of β-catenin [304]. It will be of particular interest to explore what other DNA sites MAML binds to and what other signaling pathways MAML is associated with. In **Paper IV**, our transcriptomic analysis was limited by the heterogeneity in the liver samples. For example, being able to pinpoint differences in the cholangiocytes and their progenitors would be helpful in understanding cholestasis in Alagille patients. Therefore, the use of sc-RNA seq will be advantageous in further exploring the detailed transcriptomic and cell population changes in Alagille patients. Lastly, additional investigation is needed to piece together the opposing results of WAP-Cre lineage activation of NICD and the WAP promoter driven NICD from a previous study. A more detailed time tracing (*i.e.* introducing a Dre or Flp system to only trace PI-MECs), may address whether the tumour grows from a remnant WAP cell lineage with hyperactive Notch.

The context dependent nature of Notch signaling remains one of the greatest question in the field. However, there is limited study on a unifying principle. Undeniably, one may argue that the vast differences in epigenetic landscape and interacting partners of entirely different contexts will unsurprisingly lead to the different outcomes. However, Notch signaling often exhibits opposing roles even within the same lineage development in a sequential manner. These could be useful models to study how Notch could quickly switch its role. Combining transcriptomics, CSL-, NICD ChIP-seq, chromatin structure determination and epigenetic landscape profiling in such context will definitely shed light

on the DNA side of the question. On the other hand, improved techniques in mammalian-2-hybrid systems [305] and genome-wide CRISPR-screening [306], combined with Notch reporter systems, could identify important interacting partner, interrogating at the protein side of the question.

How different Notch receptors respond differently remains unknown. In **Paper II**, we noticed differences in Notch1 and Notch2 functions in the medulloblastoma cell line. The negative results of the study where the N1ICD and N2ICD in mice were genetically swapped illustrates that the mere sequences of ICD may not sufficiently answer the question in the *in vivo* context. An alternative explanation is a gene-dosage effect, where the outcome depends on the combined NICD levels generated by the different Notch receptors. Another hypothesis is that the NICD has specific modifications according to the NECD. For example, different NECD may recruit different interacting partners, leading to different modification in the NICD. However, this direction is largely unexplored. With CRISPR-Cas9, it is much easier to specifically study the Notch receptors one at a time by removing interference from other Notch receptors. It is also easier to study how mutations alter the endogenous gene-dosage, which was previously difficult to achieve, as most previous studies were done with overexpression.



"*A Simple Touch*", Sunny Tsoi, 2020. Inspired by the De Stijl (Neoplasticism) art movement and canonical Notch signaling.

Popular Science Summary

Introduction

Over the billions years of our entire history of time, between lightness and darkness across billions light-years of our universe, there is one pale blue dot [f]. Everything on this pale blue dot was once forgettable star dust. Yet, in our pale blue dot, the star dust thrives as stardust crusaders [g], surviving and evolving with the song of life. “Perfectly balanced, as all things should be.” [h] 'Twas the perfect balance of environmental conditions that made us. 'Twas also how we stand against the ever-changing environment, to maintain a balance by reacting, regulating and relating to others, that made us. A human body is made up of more than 30 trillion “cells”, the basic unit of life, corresponding to various functions such as thinking, defense, and pumping your blood, after their kinds. “No man is an island”[i], so is no cell. Not only do we have to balance with the environment, our cells have to cooperate and balance among themselves.

Cellular communication: signaling

The Chinese word “Chung Yung” (中庸), in English the “doctrine of mean”, (or the strikingly similar Swedish word “lagom”), briefly represents the wisdom of being “just right” - not too much, not too less. A deeper meaning of Chung Yung is to do the right thing as who you are and at the right time. In living organisms, one key to balance is “cell signaling”, the communication of cells among themselves and to the environment. Just as a machine or a railway system needs a signaling system, our body also needs a signaling system. If the signaling of a railway is compromised, delay or even accidents would occur. Likewise, if cell signaling is compromised, the internal balance will be broken and diseases will occur. For instance, too much growth signal at the wrong time could possibly lead to cancer. In fact, many causes of cancer are signaling related. On the other hand, inadequate signaling could lead to the underdevelopment of important tissues and organs.

There are a few types of signaling in machines: contact-dependent, such as a button of your computer; long-ranged, such as a cable sending electric signal; wireless, such as bluetooth on your phone. Similarly, there is a great variety of types of cell signaling: long-ranged electric signaling in your nervous system; hormones in your blood; contact dependent Notch signaling, which is the key topic of our story. A functioning signal system must have intact components, such as an antenna or a button to receive signal, some cables to transmit signal, and a remote control to send signals. Comparably for

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[f] *Pale Blue Dot* is a photograph of the earth taken by Voyager 1 space probe 6 billion km from earth. It inspired astronomer Carl Sagan’s book with the same name.

[g] Stardust crusader is the title name of the Japanese manga “*JoJo's Bizarre Adventure Part-3*” (Hirohiko Araki, 1989).

[h] A signature movie line from from “*Avengers: Infinity War*” (2018) by the main antagonist Thanos, who wish to wipe out half of the lives in the universe to make balanced world.

[i] From the poem *Meditation XVII* by 17th Century British poet John Donne

cells, where we call a signal receiver as “receptors” and physical signals as “ligands”. If a door knob is a “receptor”, your hand would be a “ligand”. Equally, if a vending machine’s insert slot is a receptor, the coin would be a ligand. In addition, during the relay of the signals, there are many intermediate molecules that are very important to process or amplify the signals.

The components of a machine is manufactured and assembled according to a blueprint, so are the components of our cells. The blueprint in our cells is written in a language called DNA. Instructions for the making of one component is called a “gene”. The whole book of blueprint is called a “genome”. It contains all the instructions on how you can develop from a sperm and an egg to a complete individual reading this thesis. Just as a novel series could be broken down into different volumes, our genome is divided into different volumes. We have two collections of books, each with 23 volumes, in total 46 volumes. One set is from our father, the other from our mother. We call the physical form of these individual volume of “book” a chromosome (chroma means colour in latin). If there is an error in a blueprint, broken or faulty components may be produced. Such an error in the genome (a mutation), broken or defective cellular components may be constructed. This could lead to diseases such as inherited diseases and cancer.

The orchestral role of Notch in life and death

Notch signaling is the key topic of this thesis. It is a contact-dependent signaling, that a cell must “kiss” another cell in order to send a signal. The receiver of Notch signaling is a “Notch receptor”, locating on the outermost layer of a cell and facing outward, like a nun-chuck sticking out of the cell. Conversely, the sender of Notch signaling (including Jagged and Delta-like), locates also on the outermost layer but of another cell. Upon the “contact” of the receiver and the sender, Notch signaling is activated. This “contact” has a long evolutionary history. One can find Notch signaling from simple jellyfish like creature to insects to human. If Notch is defective in a fruit fly, there will be notches on their supposedly smooth wings, thus the name “Notch”. Notch plays a role in the development of almost all organ-systems, and is found to be related to cancer and how aggressive a cancer could be, orchestrating life and death. The importance of this “contact” often makes me think of the contact of God and Adam in “*Creation of Adam*” by Michelangelo. Therefore, I use “Notch Signaling Requiem: Orchestral Role of Notch Signaling in Cancer and Developmental Disease” as the title of my thesis, in order to investigate the roles of different components of Notch signaling in various types of diseases.

The discovery of Notch

The story of Notch started in fruit flies. In the beginning of the 19th Century, scientists were still unaware on the mechanism of inheritance. It was only speculated that chromosomes are responsible for inheritance. To study how chromosomes may contribute to inheritance, they often use fruit flies for experiments. Fruit flies are ideal because of their short life cycle of 10 days. Also, a fruit fly only possesses 4 pairs of

chromosomes, making analysis far easier. The "father of modern genetics" Thomas Hunt Morgan started a fruit fly experiments by pairing up flies with various traits, for example, red eyes or white eyes, big wings or degenerated wings, and recorded what their offspring look like. Morgan studied the mechanism of inheritance by chromosomes and was awarded the Nobel Prize in Medicine or Physiology in 1933. "Notch" is one of the traits he studied, since the notched wings are easily visible, making it easy for Morgan to observe and analyze.

Notch: more than just a "notch"

Does Notch only play a role on notched wings? Scientists discovered that male flies carrying the Notch mutation will die as embryos. This indicates that Notch is essential in life. In 1930s, Donald Frederick Poulson started his research in Yale University. During that time, geneticists in general only investigate the role of genes in adult traits. Poulson was among the first to focus on the dead fly embryos instead of the living ones. He discovered that these embryos cannot grow skin but grow a lot of nervous system like cells. If one wish to reimagine it as a human, that would be a monster with a huge brain but no skin! This was the first time a scientist associated genes to development. Unfortunately, Poulson may not have received sufficient recognition. In 1995, scientists were awarded the Nobel Prize for their discovery on genetic rules of early development. However, Poulson passed away in 1989 and could not share this honour. Nevertheless, Poulson's work sparkled that glorious "developmental biology", which later enter its golden era in the 1980s-90s. Many great discoveries, experimental techniques and molecular tools came from developmental biologists. It also gave rise to new fields such as stem cell biology, regenerative medicine and evolutionary developmental biology. Nowadays, the focus shifted to stem cells and regenerative medicine. Many people believed that developmental biology is in decline, as there is less funding and spotlight from the public or peer scientists. However, the emerging fields such as stem cells and regenerative medicine essentially trace back their knowledge to developmental biology. To know regeneration, one must know generation.

Notch and human cancer

In 1980s, with the new techniques in DNA analysis, scientists can "translate" and understand the instruction of the DNA. Spyros Artavanis-Tsakonas and Michael Young's group separately analyzed the Notch gene. They found that it resembles a "receiver" (receptor) on the outermost layer of the cells. Later, Artavanis-Tsakonas also analyzed and confirmed many components in Notch signaling, such as the signal sender "Serrate" and relay component CSL. In 1991, scientists discovered a peculiar gene in three leukemia patients that surprisingly looked like the fruit fly Notch gene. It was very encouraging, as at that time, it was one of the first examples of how researching on flies could be informative for understanding human diseases. More than ten years later, Jon C. Aster found broken copies of Notch in more than half of the leukemia patients, confirming that Notch is important in leukemia.

We now know that cancer is not a homogenous disease, but rather a collection of different diseases. Leukemia differs from liver cancer, and breast cancer differs from lung cancer. Even within one type of cancer, such as breast cancer, there are many subtypes. This is why some cancers are relatively simple to treat, while some are difficult. Some are drug resistant, while some readily relapse. This is mainly because they originate from different cells, and have different mutations in their blueprints. As time goes by, there might even be more and more mutations, making them even harder to treat. Malfunction of Notch signaling components often relates to how bad the cancer has developed.

How does Notch work?

How does Notch work? As mentioned, Notch is a receiver located at the outermost layer of a cell. Facing the environment is the outer component of Notch, responsible for receiver signals. Facing inside is the inner component of Notch, responsible for relaying the signaling. One inside, one outside, almost like a pair of door knobs with an inside and an outside knob. When Notch is activated, it is like someone turning the outer door knob. In contrast to a real door knob, upon opening, Notch does not turn, but instead gets the inner knob cut and ejected into the room. (This happened to me when I was living in a small cabin in Huddinge in a cold winter. The door knob broke and was ejected! I was trapped in the kitchen!). This liberated inner component will then head to the "brain" of the cell – the nucleus, where all the blueprints are located. The inner component combines with partners such as Mastermind (such a cool name) and a blueprint reader called CSL. Thus, the cell knows what to do and what blueprint to use in response to the signal.

My research: Notch and aggressive cancer

In **Paper I**, I use a new technique called CRISPR-Cas9 to genetically modify some breast cancer cells. I destroyed the component CSL and found that the cancer grew much faster and grew much more blood vessels to get nutrients. This is fitting the clinical observation, that 30% of patients with severe breast cancer have a broken CSL. Unlike normal cells, cancer cells enjoy a low oxygen environment. Sometimes they can even become invincible to radiotherapy under environment with no oxygen. We uncovered that the cancer cells with broken CSL behave like they are out of oxygen, even when there was oxygen. It is like they are a person pretending to be drowning on land, only that they actually love drowning. This "pseudo-low-oxygen" state may be why they form a such aggressive cancer. Continued with **Paper II**, we discovered that actually in many different cancers, Notch can control this "low-oxygen" signal. These two papers are the first to report such a link between Notch and oxygen level. Our results help in understanding why cancer can adopt a "low-oxygen" state, and provide insights into how to treat cancer patients.

My research: Notch and inherited disease

In contrast to **Paper I** which presented the importance of CSL in breast cancer, **Paper III** exposed that CSL is dispensable in some occasions. We removed CSL in blood cells of mice, and realized that the mice live happily with no problem in their blood. In normal conditions or in conditions that required quick blood replenishment, the mice still had no problem at all. On the other hand, in **Paper IV**, we explored more than blood cells, in fact the whole mouse. In the mice, we mimic the mutation of the signal sender Jagged1 in human Alagille syndrome patients. We uncovered that these mice have similar symptoms to the human patients, for example having problem in the heart, the eyes, the face, etc. They also showed complications in the bile ducts, causing skin colorations (yellow hue) in babies and bile accumulation, which affect human patients the most. By studying these mice, we gained better understanding of the patients. We also compared the liver of mice to that of Alagille children in Hong Kong, discovering commonalities among them. This advances our understanding in the disease, and the development of possible treatment.

My research: Notch and the origin of breast cancer

Why do people get breast cancer? We explored one of the possibilities. In **Paper V**, we tried to create a super Notch signal in the mammary (breast) tissue of mice, to see whether it will lead to breast problems or even breast cancer. Distinct from many organs, human breasts develop mostly during the puberty. They also further develop during pregnancy and undergo respective regression after weaning. We tried to learn about the role of Notch during and after breastfeeding. We created a super Notch signal in the mammary cells after pregnancy, and observed that the mothers failed to feed their babies. (Their babies survived with foster mothers). Some of the mice even developed breast cancer. To trace the origin of these cells, we found the cancer came from the mammary cells with super Notch signal. Previously, people thought Notch only affects the surrounding environment. Here, we demonstrated that Notch can induce cancer in the same cell as it is expressed in. We collected these mammary cells, surveyed individual data of each single cell and analyzed them together. Using artificial intelligence techniques, we discovered these cells were not much different from that of normal mice. Rather, we only noticed imbalance among these cells. This came back to our original theme “balance”. Even an imbalance may cause a serious disease.

Thoughts and conclusion

Above is my research on the song of life and death of Notch. I studied how Notch is related to life (development) and how Notch is related to death (cancer). In my odyssey of this PhD, I had gains and I had losses. My research results were humbly acceptable, contributed to various fields and involved different techniques. Although it may not be as astonishing as my ambitions when I first embarked on my PhD (apparently most first year PhD students want to earn a Nobel prize), I still considered myself lucky to be able to contribute to science. With all the weekends and overnights; grit and anxiety, I earned the famous “Permanent Head Damage” (the true meaning of a PhD), but also willpower

and problem-solving abilities. Among all, the bravery to face problems head on, is the most precious trait I had acquired. This is not an easy task, but I am still learning and improving. As I always say, I am pursuing a doctor of "philosophy" (the love of knowledge), not a doctor of "science". Therefore, broad knowledge and critical thinking weigh more than mere professional knowledge, which is of course also very important. I am very grateful I met many great teachers and fellows whom guided me in science, culture, history, language, music, fighting, and many more. I am still in the darkness of foolishness, but I considered this as the beginning of my journey. Now I am working on heart development and regeneration, hoping to find insight in heart repair by comparing mice, newts and human. Nevertheless, "Man is born free" (Rousseau, 1762), we are not bound in academia only. There are still a lot of other meaningful pursuits in life. I do not know how long I will stay as a full-time scientist, and of course have thought of the regret I would have if I made a pause in science. However, I want to conclude with this line I composed, "Does the science make the doctor, or does the doctor make the science?" In the past, I thought a title of a person is mere lip-service, vague and empty. Now I wish, after my proper defense, wherever I go, I could bear the name of a Doctor, to encourage and to constrain me to keep my development and integrity. To be a doctor who lives the motto of my home university; the Chinese University of Hong Kong – 'Through learning and temperance to virtue' (博文約禮).

"No. It's Doctor Strange. Not master strange. Not mister strange.
Doctor Strange." - 《*Doctor Strange*》2016

科普概述 Popular Science Summary (in Chinese)

引言

恢恢宇宙， 漫漫時間，跨不知幾千億里幾千億年的光明與黑暗間，有一暗淡藍點。這暗淡藍點，正是我們的地球。在浩瀚宇宙中，本不過是轉眼即逝的渺渺星塵。 然而其上星塵，卻化作星塵鬥士，演化成芸芸萬物，勃勃生機 。《復仇者聯盟：無限之戰》中的薩諾斯 (Thanos) 力求平衡：「完美平衡，凡事本應如此。」(Perfectly balanced, as all things should be.) 正正是各種環境條件的匹配與平衡，造就了萬物發生的機會。而萬物亦致力在萬變的環境中反應、控制、合作，從而維繫平衡，生生不息。我們的身體，由三兆個細胞組成，各從其類，分工合作，有些負責思考，有些負責防衛，有些負責供氧，崗位數之不盡。「沒有人是孤島」[j]，也沒有細胞是孤島。人類不單要與外界平衡，細胞之間，也要平衡。

細胞訊號

這就像華人常講的「中庸」，通俗解是不偏不倚，無過無不及（瑞典語也有 lagom 一詞，但求平均，不要出眾），但也強調「誠」，發乎天性，要在適合的時地做適合的事。「誠者，天之道也。」（《中庸》），生物界也如是 。細胞要達至平衡，主要靠他們之間並對外的溝通，我們稱這溝通為「訊號」(Signaling)，就如機器和鐵路用到的訊號。鐵路訊號出錯，可能導致延誤，甚至車毀人亡，而細胞訊號出錯，則會破壞平衡，引致各種疾病。例如，過量的生長訊號，可能導致細胞不斷增生，甚至形成癌症。事實上，很大部份導致癌症的病變，都是和細胞訊號有關的。相反，過少的發育訊號，可能導致發育不良，器官功能不健全，甚至缺少部份器官。

機器的訊號，有各種類型：有接觸式的，例如按按鈕開電腦；有遙距的， 例如用電纜傳送電子訊號；也有無線遙控的，例如用藍芽的無線電通訊。細胞訊號亦是多姿多采：有遙距的腦神經訊號，有在血液中傳訊的激素（或作賀爾蒙），而全接觸式的 Notch 訊號，就是本博士論文的主角（Notch 是刻痕的意思，容後解釋）。健全的訊號系統，就必要有全備的零件，例如接收訊號的天線、按鈕、傳輸訊號的電纜、發出訊號的遙控器等等。細胞亦然，接收訊號的接收器，稱作「受體」(Receptors)，就像是細胞的天線、按鈕、或者收件箱；刺激受體以啟動訊號的分子是訊息發送器，稱作「配體」(Ligands)，就像是按

[j] 出自十七世紀英國詩人唐約翰(John Donne)的詩《Meditation XVII》

按鈕那隻手指、飛鴿傳書的書信等；而處理訊號的過程，也涉及很多不同的分子，對訊號傳遞舉足輕重。

正如機器的零件要在工廠按照藍圖生產和組裝，細胞的零件，也需要按藍圖生產和組裝。人類細胞的藍圖，是以 DNA 寫成，每段零件的說明，我們稱為基因(Gene)，而整個人體的生命藍圖，我們叫作基因組(Genome)，記載了你如何由精子卵子變成現在高大威猛或者亭亭玉立的人。正如一套書叢可以分出各種冊數，我們的基因組，也分成兩套共四十六冊， 就是廿三對染色體(chromosome)，每套分別來自父親和母親。藍圖出錯，自然會做出錯的零件，所以基因突變（mutation）也可能導致細胞零件錯誤，破壞了平衡，引致遺傳病和癌症等等。

指揮生與死的 NOTCH

Notch 訊號通路(Notch signaling)，本博士論文的主角，是觸碰型訊號通路，需要細胞和細胞之間「親吻」，才可傳遞訊號。負責接訊號的 Notch 接收器，位於細胞膜（細胞最外層）上，對著外界，有如對外的雙截棍。而負責發出訊號的配體（包括 Jagged 和 Delta–like），位於其他細胞的外層。你一觸，我一碰，就啟動了 Notch 訊號通路。這「觸碰」，源遠流長，由最簡單的水母類生物，直到複雜的人類，都可以找到 Notch 的蹤影。果蠅的 Notch 突變的話，會令其本應圓滑的翼上出現坑痕(notch)，因而得名。Notch 幾乎對每個器官的發育都很重要，Notch 的變異也和很多癌症和末期癌症有關，可謂掌控生與死。這「觸碰」影響之深廣，令我常想起米高安哲羅的名畫《創造亞當》，是維繫生物生與死的重要觸碰，有如上帝和亞當的觸碰。故本博士論文定了《刻痕安魂曲：Notch 指揮遺傳病和癌症的生死樂章》為題，以探討 Notch 訊號通路的各個零件對疾病有何角色。

NOTCH 的發現

Notch 的故事，要由果蠅說起。二十世紀初，科學家還未很清楚遺傳學的實際機理，只猜測生物特質是以染色體遺傳 。為了一窺究竟，證明染色體的遺傳假說，他們使用果蠅做實驗，因為果蠅生長得快，十日時間就可以由蟲卵變成蟲，也只有四對染色體，方便分析。現代遺傳學之父莫爾根(Thomas Hunt Morgan)，將有各種特質，例如紅眼白眼大翼小翼的果蠅，以各種組合互相交配，統計牠們後代的特質，從而分析遺傳的原理。 莫爾根藉此確認了染色體的遺傳機理，最後獲得 1933 年諾貝爾生理醫學獎。Notch，正正就是莫爾根分析

的其中一個特質。因為 Notch 突變導致的坑痕非常易見，這大大幫助了莫爾根的分析。

NOTCH：不僅是坑痕

Notch 僅僅影響翼上坑痕嗎？科學家發現， Notch 基因突變的雄性果蠅在胚胎時期會夭折，說明 Notch 對生命非常重要。 在 1930 年代，剛從加州理工學院完成博士的保森(Donald Frederick Poulson)到了耶魯大學展開果蠅研究。那時候，普遍遺傳學家都只會研究基因與成年特徵的關係，並無在意基因和發育的關係，保森卻集中研究那些夭折的果蠅胚胎。他發現原來基因掌控了發育過程，那些 Notch 變種的夭折胚胎，大部份細胞都變成了神經細胞，卻發展不出外皮，想像是人的話，就像是個只有大腦卻沒有皮膚的怪物！這是首次有科學家將基因連繫到胚胎發育，可惜，他這方面的貢獻並無受太大關注。直到 1995 年，其他科學家才因為研究胚胎發育基因而得到諾貝爾生理醫學獎，而保森早在 1989 年就與世長辭，未能共享這榮譽。無論如何，這也算是發育生物學的先河，在 1980–90 年代更加是黃金時代，很多重大的發現、實驗方法和分子工具，都是來自發育生物學家。發育生物學更衍生了後來的幹細胞生物學、再生醫學、演化發育學等等。如今，科學界的焦點已轉到幹細胞和再生醫學上面。無奈，很多人都認同，自 2000 年代後發育生物學就已式微，風光不再，沒有眾多的研究資金，也得不到科學界和外界的鎂光燈。其實，發育生物學仍然重要，未知生，焉知再生。(To know regeneration, one must know generation)

NOTCH 與人類癌症

1980 年代，隨著 DNA 序列分析的普及，科學家可以開始「翻譯」和「解讀」生命的藍圖。查剛拿（Spyros Artavanis-Tsakonas）和楊米高（Michael Young）各自的團隊分別分析了 Notch 基因，發現 Notch 很有可能是位於細胞外層的接收器（受體）。一鼓作氣，查剛拿也分析並證實了很多果蠅版本的 Notch 訊號通路的組件，例如發送訊號的 Jagged（鋸齒的意思），通路零件 CSL 等等。在 1991 年，科學家發現有三位血癌病人都有一個怪異的基因，成份和果蠅的 Notch 很像，後來發現是人類本來正常的 Notch 突變後的致癌基因，說明 Notch 很有可能對人類癌症非常重要。原來，研究果蠅基因，是可以理解人類疾病的，這令當時的科學界非常振奮 。十多年後，雅士特(Jon C. Aster)在超過五成血癌病人內找到 Notch 的突變， 並證明了 Notch 對血癌的重要性。

原來，癌症並不是一種病症，是很多種不同的病症，血癌和乳癌不同，肝癌和胃癌又不同，甚至同一種癌症，例如乳癌，都分很多亞種。這就是為何有些乳癌能醫，有些乳癌很難醫，有些會有抗藥性，有些會容易復發。種種不同，都是源於有不同的基因突變，以及起源自不同的細胞。甚至隨著時間推移，突變會越來越多，適者生存，最惡毒的癌細胞活下來，導致癌症越來越惡性。而 Notch 訊號通路的零件出錯，很多時候都和癌症的惡性相關，在我的研究也有所涉及。

NOTCH 的原理

Notch 是如何運作的？如上述所言，Notch 是位於細胞最外層的接收器。Notch 有外露的部份（體外結構; ECD），是負責接收訊息的部份，形如雙截棍，也有細胞內的部份（體內結構; ICD），負責在細胞內傳遞訊號。一外一內，就好像房門的門柄。當 Notch 被訊息發送器啟動，就好像有人來握手把開門，啟動一連串的機理。和門柄不同的是，Notch 的反應不是旋轉，而是斷開。啟動時，Notch 的體內結構(ICD)會被像剪刀的東西切開，想像你一扭外面門柄，房間內的門柄就突然裂開並飛出去（我在以前住的林中小屋中親身經歷過！還因此被困在廚房裡。）。這體內結構從細胞表層釋放了，就會跑到細胞的中樞（細胞核），即 DNA 的所在地，並和其他分子六神合體，包括 Mastermind（首腦。對，基因名字有時很帥的。），和負責閱讀生命藍圖的閱讀器 CSL，從而啟動指定的下游目標行動。

我的研究：Notch 與惡性癌症

我在*首篇論文*中，我用近年的新技術 CRISPR–Cas9[k]，基因改造了乳癌細胞，破壞了閱讀器 CSL。我們發現破壞閱讀器 CSL 會導致乳癌生長的更快，並且更容易生增血管吸取營養。 這和臨床的研究吻合，因為在三成的乳癌病人樣本中，CSL 也是被破壞或干擾了的。癌細胞和正常細胞不同，很多時候，都更適應，甚至偏愛無氧氣的環境。無氧狀態更會令某些癌細胞對放射治療免疫。我們破壞了 CSL，癌細胞會呈現「假缺氧」的狀態，即是在有氧環境下也啟動了無氧模式，也許就是令癌細胞更惡性的原因。承接*首篇論文*，我們在*第二論文*中發現了 Notch 訊號通路能夠在多種癌細胞中干預細胞的缺氧反應，再度證明了 Notch 能強化細胞的缺氧狀態。在這兩篇論文前，證明 Notch 能影響缺氧反

[k] 用逆向工程，將細菌防衛系統改造成科學家的工具，可以快速修改基因。2018 年中国研究員賀建奎聲稱以此技術製造了人類使上首兩名基因改造人。

應的研究並不多，這些結果有助了解為何癌症如何適應缺氧狀態，並對療法運用提出了新見解。

我的研究：Notch 與先天性遺傳病

相對*首篇論文*證實 CSL 舉足輕重的地位，*第三論文*則證明了原來 CSL 在罕有的情況下是無關緊要的。我們在小鼠的血液幹細胞中移除了 CSL，卻發現小鼠的紅血球和血小板完全沒影響，無論在正常情況製造血液，或者在損血情況下補充血液，都沒有影響。*第四論文*，則不只針對血液細胞，是針對整隻小鼠。我們在小鼠上模擬了人類遺傳病亞拉周症候群(Alagille Syndrome)中，其中一種訊號發送器 Jagged1 的突變，發現此小鼠在多個器官都有和病人類似的症狀，包括肝、心、眼、面、內耳的疾病等（因為內耳有問題，此鼠經常點頭，我們叫牠們做「點頭鼠」。）。特別是膽管的延緩發育，導致黃疸病、膽管閉塞等最影響亞拉周病人的症狀。研究此鼠，就有助研究病人，了解如何幫助他們。我們也在香港取得亞拉周病童的肝臟樣本，和小鼠肝臟的比對下，發現了很多共通點，有助了解疾病機理，藥物和治療研究。

我的研究：Notch 與乳癌發展

乳癌的成因為何呢？我們探索了其中一種可能。 我們在*第五論文*中，嘗試在小鼠的乳腺啟動過量的 Notch 訊號，看看會否導致發育不全，甚至癌症。和一般的器官不同，乳腺主要在青春期發育，在懷孕和哺乳也會再發育，並在哺乳結束後稍稍萎縮。我們想知道，哺乳過程中，Notch 有否重要影響。我們在小鼠懷孕後，在乳腺細胞啟動過量的 Notch 訊號，並發現其嚴重影響乳腺發育，甚至令母鼠無法餵奶，初生小鼠需要在奶媽的養育下才能成長。其中一些母鼠更發展出乳癌，追查下發現這些癌細胞都是源自懷孕後發育的乳腺管道細胞，甚至能蔓延到肺部。這和以往的研究不同，他們認為 Notch 訊號只會影響週邊環境，間接幫助癌症發展，我們卻證明了過量 Notch 訊號能直接導致乳癌發展。我們也提取了小鼠的乳腺細胞，進行單細胞分析，將每個細胞的資訊分別收集然後再整合，再用人工智能分類和分析，發現其實正常小鼠和變種小鼠的細胞分別不大，卻只是細胞比例失去平衡。這呼應我首段所述，平衡是非常重要的，失衡，甚至可能和癌症相關。

結語和感言

以上就是 Notch 的生與死之歌，我研究了 Notch 對生（發育）的影響，也研究了 Notch 對死（癌症）的影響。走過這段博士旅程，有得也有失。研究成果算是可以，也涉獵不少範籌和技術，儘管未如開始博士時那雄心壯志，也算是為科學盡了一分力。捱過多少個週末和通宵，承受過幾多精神壓力和傷害，贏得了永久大腦損害(Permanent Head Damage；PhD)，也練到了意志力和解難能力，更重要是面對困難時的氣量，這不容易，也仍在學習。我常說，我讀的是博士，不是專士，是「哲學」博士(Doctor of Philosophy)，不是科學博士，故廣博見識和批判思考比專業知識更為重要。有幸在博士期間也遇到不了前輩，教導了我文化、政治、歷史、語言、音樂、武學等知識，不算廣博，但也算開導了追尋自我的前程。如今我從事心臟發育與再生研究，比對小鼠、蠑螈和人類的心臟，看看能否找出心臟再生的鑰匙。然而，人生而自由(盧梭，1762)，人生並不局限在學術界的圈子，也有太多志事要追求，我不知會再從事科學研究多久，我也有考慮到離開暫別科研會帶來的遺憾。容我以這句做總結：「究竟是科學成就了博士，還是博士成就了科學？」(Does the science make the doctor, or does the doctor make the science?) 以往我以為名銜只是虛浮，但願成功答辯以後，無論到何處，博士名銜也成為我的約束和鼓勵，進德修業，也秉承母校香港中文大學的校訓－「博文約禮」。

“No. It’s Doctor Strange. Not master strange. Not mister strange. Doctor Strange.” – 《奇異博士》2016

Acknowledgements 鳴謝

If you are reading this now, I am sure you are an important support of my journey on this great odyssey. Let me first express my gratitude to you, for all your support and for reading my first written book. I try to make this book welcoming to everyone, by including my all-time experience in some molecular tools, and a popular science summary in both English and Chinese. I hope you find my book helpful and interesting, whether you are a biologist or not.

I could not believe I am writing this now, and all the sweet or sometimes bitter memories remind me such a wonderful journey I have had in Karolinska Institutet. The first day I was in KI, I was actually sneaking into a DBRM seminar, hoping to get to know someone and get into KI. Little do I know many of the people in the room will become my friends, my colleagues and my mentors. I would not have made it if I did not have met such great people and such immense support. I am grateful to have so many supportive, talented and inspiring people around me. This text is just merely a tiny part of what I want to express to all of you. Allow me to express my gratitude with the limits of my words.

I must first thank my supervisor **Urban Lendahl**, who led to the beginning of my whole journey in Karolinska Institutet. I still remember the first email I sent and the invitation received from you when I was still an undergraduate exchange student, such a new arrival in Europe, an infant in science and a frog in a well. You gave me full trust and allowed me to work as a project student, eventually giving me the precious opportunity to start a PhD. You have been my role model in science - compassionate and wise, knowledgeable and ever-learning, critical but generous. Because of you I embarked my journey on Notch and it is now my all-time favorite pathway and the super-biasedly-emphasized pathway when I am giving lectures to KI students. Thank you for being patient with me and trusting me in my process of learning, especially in my greatest weakness in paperwork and social etiquette. I also enjoy all our talk of science and non-science when we are not discussing projects. Your inspiration and guidance will be my lifelong precious.

Another very important person in my academic life is my co-supervisor **Ken Chien**, a.k.a. “Boss”, who I mostly work with during these six years. You are a role model of me, decisive and bold, intelligent and thoughtful. Thank you for giving me full support and giving me flexibility to experiment with different things. Every of your speech sparkled my fire in science. Outside of science, you are a caring person to me. I will not forget your trust in me in when I was my lower times. From science to biotechnology to therapeutics, I cannot express how much bigger the world I see because of you. I hope one day to be as inspiring and influencing as you.

Lendahl Lab – I am grateful to meet all kind people during my time in the Lendahl lab. **Shaobo** is my daily mentor and the first person to guide me in KI. Thank you for

treating me almost like a son during these times. You always think of my best and stand by me. I am very grateful to have you in my life. **Eike-Benjamin**, you are always there late in night so I could come and rant and harass you when I work late. I am happy with the fruitful projects and discussion we had. **Francesca**, same to you, thank you for being my daily talking buddy at the end of the day back in CMB building. Thank you for always being cheerful. **Anders**, the musician and fun guy. Thank you for inspiring me to also work on music in my PhD! **Indira**, the law and order of the lab, the opposite of me as an agent of chaos. I am happy to work with you. Thank you for **YP, Tanya** and other previous members for being good friends and good colleagues.

Chien Lab – I must say I found myself privileged as a part of the Chien lab family. I had so much inspiration, support and fun from you guys. Thank you for being patient with me when I undergo EMT and metastasize. I am not a born extrovert, and sometimes could be socially anxious. I may not be able to join all the fun times with you guys but I really like being with you all. **Elif** is the first person I must thank. You ALWAYS trust me in all circumstances, in my bright times and even when in my darker times. I am very grateful I have the chance to work with such a clever, hardworking, caring person and a good leader. You are a great colleague and a great friend. If without you I couldn't have made it. **Kylie**, my Cantonese buddy all the time. Thank you for being a great friend and a great snack supplier. I am flattered Theo thought I am his uncle! **Chikai**, my daily working buddy. Thank you for being there all the time and I am happy designing and troubleshooting experiments together. We really work synergistically! **Xiaobing**, thank you for being the savior of us all. Without you anything would not have been possible. Also great thanks to **Jesper**, who is both professional in lab and cat facts. Thank all the great people in the newt team! Thank **Chris** for being my daily science buddy, **Tamara** for being such a caring friend and helpful colleague, **Chimezie** for your everyday bright smile and amazing baking skills. Thank **Yao** for all the involvement in my mouse experiments. **Alex**, thank you for your daily German humour and your intelligence for the inspiring scientific discussion with you. **Edu**, thank you for all the goodie tips and all the fun in the lab. Thank **Nevin** for making America great again in our lab, and all the laughs and support as a pioneer member of the lab! **Makoto**, thank you for all the intellectual and fun exchange. Thank **Raffa** and **Katarina** for all the daily help in all kinds of arrangement. Thank **Mimmi** for being a nice colleague. **Fede**, thank you for being such a cheering person and inspiring person. You inspire me a lot when I am struggling with my career decision. **Whitney**, for being a good friend who accompanied me during my darkest hours. **Jiejia**, for all the "not many words but Bam here are my plasmids and advice". **Miia**, for being a great colleague sitting next to me. Great to have such athletic colleagues **CY** and **Niels**. **Erwin**, for your expertise in intrabody. Thank **Kristine** for kindly organizing nice Christmas parties in your warm house. Thank **Lance** for kick-starting all my stem cell biology techniques. I am also grateful with **Kalai**, **Rocio**, **Bingqian** and all other previous lab members that shared great times with us. Thank you all. It was really a wonderful time.

CMB/Biomedicum – I am happy I met so many great people in CMB and in Biomedicum, especially friends from A6, D6, Andersson lab, Göritz lab, Simon lab. The first I must thank is **Benjamin**, who is my high school and university classmate. I am happy we are still "classmate" as PhD students far here in Sweden. You are always to cheerful and passionate person. I enjoy all the crazy science talk with have. Talking to you often make me feel like what all the great scientists in history would do. Thank you **Simona**, who has been my friend since I started in KI. You are always cheerful and hard-working. I enjoy all the "5 minutes before my timer goes" encounters. Thank you for being such a great friend and all the deep talks and parties we had. Also great thanks to the help in my preparation for the defense. **Emma**, for always being thoughtful and motivational about science. I am happy to have you as a collaborator on the Nodder paper. Thank **Nino** for showing me all the cell culture and hypoxia techniques when I started. You are a good friend and I enjoy our time together. Thank all the fika buddies! Thank **Katrin** for the great time since you were in the Chien lab. Thank for all the supportive chat we have when we are buzzing between A6 and D6. Thank **Anna** for all the chat especially in exhausted moments. Thank **Edu**, **Soniya**, **Daniel**, **Helena**, **David**, **Tati**, **Alberto**, **Alek**, **Maria**, **Christina**, **Jan**, for all the tea break moments and off-work hours fun. Thank **Andras Simon** and **Ahmed** for being great collaborators to work with! Thank you **Matthew** for all the teaching opportunities. Thank you **Javier** for your excellent skills and immense help on FACS, and all chats on history and politics during the FACS hours. Thank everyone in the A6 quarter to provide such a nice and friendly working environment, especially **Annika** for your morning cheerful talks, **Diana** for the patience I keep buzzing you with the cryostat, **Amy** for the bouldering times. Thank **Qiaoling**, for being a nice friend and good PI to talk to. Thank **Daniel**, for all the collaboration and guidance on bioinformatics. Thank **Matti Nikkola, Lina, Margaret, Linda** and **Mia** for all the help along my study.

Special thanks to **Urban**, **Shaobo**, **Kylie** and **KK Tong** for giving suggestion and proofreading my thesis. Thank **Elaine** for the stunning cover painting and the advice on my thesis artwork. Thank **Sui** for the advice on the graphic illustration. Thank all my collaborators, especially the **Andersson lab**, **Jacobsen lab** and **Simon lab**.

My lifelong friends from Hong Kong also gave me overwhelming support through the years, especially when I was all alone here in Sweden. Thank **Fai**, for being a brother to me and all the adventure together. I am glad to have the mutual support in your on call duty and my late night. I am sure we will make a difference and become successful in life. **KTY** and **Nadia**, for being caring and honest, whipping me to be a better person. **Lydia,** for being a cheerful and smart friend. **Hasoak**, for your support and your laughter. **Moon**, for the often chat in our special times. Also thank **Gordon**, **Esther**, **Sam**, **Suki**, **Rex**, **Jody**, **Tom**, **Johnny Tam**, **Karen**, and friends I could not list all here.

Along the journey in Stockholm and Europe I met so many supportive good friends. Thank all the fellow exchange students who are my delight in life, especially **Tony**,

Calford, **Cynthia**, **Tiffany**, **Kenny**, **Matthew**, **Kitty**, **Kim**, **Curtis**, **Steven**, and **Sasa**. Also the visiting students every year, especially **Enoch**, **Christine**, **Maggie**, **Sylvia**, **Alison**, **Angel**, **Jon**, **Travis**, **Heidi**, **Deborah** and **Victor**. All the gathering, musical and theatrical times are memorable. **Caridee** and **Chantelle**, for all the great journey and fun we had. **Maru**, for being such a gentleman and talented friend. **Ka**, for the nice dinner to your place and often great chat. **David**, for your inspiring popular science books. Working holiday friends especially **Chloe**, **Sui**, **Clara**, **Veron** and **Patrick**, for all the fun dinner and cooking. I also thank my countless **readers** who support me and accompany me the days and nights at work.

It is surprising how much faith one actually needs in science. To explore the unknown, to test the untested, and to endure to uncertainty in experimental results and career, one needs a strong faith. I am grateful my faith helps me to get through all of this. I am happy to have spiritual support in the journey. Thank all my friends in the Chinese Church, especially **Jingwen**, who has been like a bigger sister to me. **Peiyao**, **Yunghui** and **Caicai**, for often caring of my well-being. **Manshu**, for being so supportive and caring. Thank all good fellows especially, **Tianqi**, **Zhihao**, **Hanfeng**, **Wang Yan**, **Wenjie**, **Yifan**, **Yafeng**, **Kaiyu** and **Ka Wai**, **Liwen**, **Mengyin**, **Xuan**, **Yu Pei**, **Ziqun**, **Nanxi**, and **Mandy**. Thank **God** for granting me faith and bravery to embark on the scientific journey, protecting me in my lower times, and accepting me despite I am a rogue and renegade person. If not all the coincidence, fortunate encounters and good people I have had, I would not have been here.

I must thank my teachers and mentors and senpais for inspiring me and guiding me on the intellectual path. My high school teachers have been my guidance and lifelong friends. Every time I went back to Hong Kong I visited them to feel home, a scientist's root. I have to especially thank **Mr. Chui** who inspired me to appreciate and understand biology, **Ms. Lee** for believing our poetic nature as a science class, **Mr. Kong** for spending time with me every of my visit, **Wingie** for always believing in me, **Ms. Lam** and **Ms. Ching** for caring about me in my lower moments. Your trust allows a small guy with a humble origin to be a man who has reached this far. Thank all my undergraduate teachers and mentors, who made me well-equipped to start a doctoral study, especially **Dr. KM Kwan** for letting me be part of Lab G96 as the start of my whole research life, **Dr. KB Wong** for being my supervisor for literature review and research design, **Dr. Jacky Ngo** for being my mentor to guide me to my overseas' study, **Andy Liu** for all the guidance on practical experiments, **Dr. MC Chu**, for pushing me to pursue my study overseas. Thank my senpais in G96, especially **Pasta** for your constant inspiration in science, **Crystal** for being cheerful and supportive, and of course my mentor **KK Tong**, who taught me almost every essential skill and technique in a research lab. Your talent and passion encouraged me to pursue a path after you. I am glad we are lifelong friends and still have constant scientific exchanges. Also happy to spend time with **Eunice** and your daughters. Lastly, I must thank **Dr. Wan Chin**, for being a role model as a scholar, and inspire me to the path of humbleness, knowledge and virtue. Be brave to bear a

hardly welcoming avatar, just to guide and stir people to seek a better path. It reminds me my favorite philosopher Søren Kierkegaard, who often impersonated in his writing, for his method of "indirect communication". Before my understanding of your choice, I was still an ignorant and arrogant person. Now I learned to be humble to appreciate the philosophical, cultural, spiritual and political aspects of life, to pursue as a well-round scholar. It's a pity we have limited personal contact, which is only one line from you – "Being rational is being strong." Yet, it is precise and concise enough, for the uneasy time I personally was facing, or our people are facing.

I cannot express how privileged I am to have **Elaine** in my life. You are an artist from Venus and I am a scientist from Mars. Thank you for painting all the colours in my life. You are my every day's motivation and inspiration. I admire your talent and kindness. Your paintings often soothe me when I am in distress. You always believe in me and can always point out my blind spot when I am clouded by thoughts and work. Our exchange in art, science and life makes me complete. Thank you for putting up with me and loving me.

Finally, I must thank my family for their full support. Despite their humble origin and limited resources, my parents always believe in me and always support me to pursue whatever I want. **Dad** and **Mum**, thank you for being humble, hard-working and kind. The most I learn from you is goodness and integrity, which is very important but not easily found in this world. You gave up so much in your life, so I could pursue my own path. My younger brother **Kit**, thank you for always believe in me and see me as your pride. I am happy we could now enjoy some adventures together which we haven't when we were younger. Also thank my **Grandma**, for your endless love and support. Everyone in my **extended family community**, without your financial support I would not be able to be here.

感謝父母養育，恩重如山，儘管生活平凡，仍全力支持我追求理想。謝謝你們對我無限信任，雖我粗疏大意，仍然信任當日的小伙子能獨自闖蕩江湖。感謝父母以身作則，教導溫馴良善，儘管我性急魯莽，常失禮失言，但仍能約以正道，尊重自省，才能走到今日，克己，剛強，望有日能為誠之者。「父母在，不遠遊。」孩兒實在有虧，願今後力爭上游，進德修業，報答親恩。也必要感謝婆婆多年照顧，使我健康成長，如今孫兒在遠方，實在有虧，願能報答。感謝祖父祖母以及各位長輩厚恩，資助我來瑞典，也特別感謝二舅父做我學術上的榜樣，四舅父的照顧，和五舅父對我厚加的支持。感謝胞弟對我的信任與厚愛，兄長不才，未能做好榜樣，也未能帶你四處遊歷，但仍珍惜少有能與你闖蕩遊玩之時，年少時鮮有機會，今日歡喜無任。願今後我能以身作則，與你互勉互勵，勇於承擔，照顧父母，一同努力，共創高峰。

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